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Recombinant Polypeptides of the Members of the TNF Ligand Family and Use Thereof

The present invention relates to polypeptides, which comprise at least three monomers of a member of the TNF ligand family as component A and at least two peptide linkers as component B, whereby the peptide linkers link the monomers of the member of the TNF ligand family to one another. Furthermore, the present invention relates to the use of these polypeptides for the treatment of diseases and for the preparation of a medication or vaccine. Moreover, the invention relates to methods for preparing and isolating these polypeptides and to the nucleic acids coding for the polypeptides, to vectors containing these nucleic acids, to host cells transfected with these vectors, and pharmaceutical compositions, containing these objects of the invention. Finally, the invention relates methods for the extracorporeal manipulation, depletion, and/or removal of components present in body fluids, e.g., by apheresis.

Members of the TNF ligand family are proinflammatory cytokines. Cytokines in general and members of the TNF ligand family in particular play a important role in the stimulation and coordination of the innate immune system and the humoral (antibody-mediated) immune response, the induction of apoptosis, synthesis of bone, formation of anlagen for hair growth, tooth growth, and sweat gland development, the anlage for lymph nodes, and many more (Aggarwal, B.B. (2003), Nat. Rev. Immunol. 3, 745-756). Defective regulation of members of the TNF ligand family, on the contrary, can lead to

numerous pathological conditions. These include, for example, septic shock, autoimmune diseases, such as rheumatoid arthritis, or neurodegenerative diseases. The tumor necrosis factor (TNF) is the eponymous and arguably the most important member of this large cytokine family.

Members of the TNF ligand family exert their action in their biologically active form as homotrimers (Banner, D.W. et al., (1993) Cell 73, 431-445). Many trimeric structures and also aggregations of a higher order (e.g., oligomers or multimers of trimers) of proteins are encountered in nature. Examples are the cartilage matrix protein (CMP), a connective tissue protein (Beck et al. (1996), J Mol Biol 256, 909-923), proteins from the collagen family, such as the Clq family, which includes Clq, collagen α1 (X), α2 (VII), the hibernation protein, ACRP30, the inner ear protein, cellebrin [sic, cerebellin ?], and multimerin (Kishore and Reid, (1999), Immunopharmacol. 42, 15-21), and proteins of the collectin family, such as the lung surfactant protein A (SP-A) and the mannose binding protein (MBP) (Epstein et al. (1996), Current Opinion in Immunology, Vol 8 No. 1, 29-35).

The assembly of proteins into a trimer occurs at the surfaces of these proteins, which trimerize in solution due to interactions, such as hydrophobic interactions, hydrogen bridge formation, covalent bonds (e.g., disulfide bridges), and/or Coulomb forces, but also due to structural motifs, i.e., characteristic amino acid sequences that bring about the formation of intermolecular supersecondary structures. In the case of members of the TNF ligand family, the three monomers in the homotrimeric structure are held together non-covalently by hydrophobic bonds. In their activated form, they in turn activate their opposite members of the TNF receptor family, which have no enzymatic activity as such. For example, TNF as a member of the TNF ligand family binds to the two membrane receptors TNFR1 and TNFR2 and mediates the trimerization of receptors or the activation of receptors already in trimer form but signal-inactive. The complex formation of receptors initiates a signal cascade, which is accompanied, inter alia, by an association of cytoplasmic adapter proteins (Wajant, H. et al (2003), Cell Death Differ, 10, 45-65). The trimeric structure of TNFR1 and TNFR2 forms in such a way that the receptors each bind in the interspace between two of the three TNF monomers of the TNF homotrimers

(Banner et al. (1993), *supra*). It clear from this that both TNF and the other members of the TNF ligand family are biologically active only in their structure as homotrimers.

Because of their function, the members of the TNF ligand family or their membrane receptors can be used variously for the treatment of numerous diseases, such as infectious and inflammatory diseases, metabolic diseases, diseases based on defective regulation of apoptosis, neurodegenerative diseases, and many other diseases. Their use in the treatment of cancer diseases plays an especially important role, because members of the TNF ligand family are usually substances exhibiting antitumor activity. To be noted in particular in this regard are TNF itself (Eggermont, A.M. and ten Hagen, T.L. (2003), Curr. Oncol. Rep. 5, 79-80), TRAIL (TNF-related apoptosis-inducing ligand), also called Apo 2L (Weley et al. (1995), Immunity 6: 673-682; Petti et al. (1996) J Biol Chem 271: 12687-12689), and FasL. *In vivo* studies, however, showed severe systemic adverse effects of TNF and agonists of the Fas receptor and *in vitro* studies also indicate similar toxic effects for certain TRAIL preparations (Jo et al. (2000) Nat Med 6: 564-567, Ichikawa et al. (2001) Nat Med 7: 954-960; Ogasawara et al. (1993) Nature 364: 806-809). For example, agonistic antibodies to Fas, the FasL receptor, exhibited an extremely hepatotoxic effect (Ogasawara et al. (1993), *supra*). For this reason, in the case of Fas-activating ligands/agonists, clinical use has been ruled out thus far for reasons of safety. Because of the considerable importance of TNF, TRAIL, FasL, and other TNF ligand family members in this field and the adverse effects associated with their administration in the form of clinical systemic dosing, however, several approaches were pursued to minimize these adverse effects. (Eggermont, A.M. and ten Hagen, T.L. (2003), Curr. Oncol. Rep. 5, 79-80.)

Thus, for example, WO 02/22680 describes fusion proteins, which enable a directed and tissue-specific or cell-specific effect of cytokines by fusion of the cytokine with an antigen-binding antibody. The result achieved in this way is that the cytokines exert no effect on tissues or cells not coming into contact with these fusion proteins and that adverse effects on these tissues or cells are reduced.

DE 102 47 755 discloses an antibody-independent system, which also enables a directed and tissue-specific or cell-specific action of the cytokines. This refers to fusion proteins, in which the activation of the protein segment, contained therein, with biological function occurs via its binding to a cell-surface molecule binding domain, which represents another protein segment of the fusion protein. Apart from a reduction of adverse effects on non-target tissue, it is also possible to use this system advantageously for cell-surface molecules on the target cells, for which no antibodies or antibodies with a too low specificity are available.

Apart from the noted adverse effects, however, it is also very problematic that the active homotrimers of the members of the TNF ligand family dissociate in dilutions, and this means in physiologically meaningful concentrations as well. This dissociation is in fact basically reversible, but the protein rapidly loses its bioactivity, because it denatures. It is assumed that this denaturation occurs via the step of unstable monomers (Smith, R.A. and Baglioni, C. (1987), J. Biol. Chem. 262, 6951-6954; Narhi, L.O. and Arakawa, T. (1987), Biochem. Biophys. Res. Commun. 147, 740-746).

Because of similar observations with TRAIL, which is especially labile, efforts were made to achieve a stability of the protein by addition of a leucine zipper as a trimerization module (Cha, S.S. et al., (1999), Immunity 11, 253-261). In its native state, TRAIL is stabilized by a zinc ion, which sits in the center of the trimeric ligand, and which is coordinated by cysteine residues (Hymowitz, S.G. et al. (2000), Biochemistry 39, 633-640). It can be a disadvantage here, however, that the addition of the leucine zipper not only achieves increased stability, but could also have a detrimental effect on other properties, e.g., structural properties such as structural changes, activity rates, or physiological properties.

There is a need, therefore, to increase the stability of active cytokines, particularly members of the TNF ligand family, without detrimentally affecting their native properties by a change in their structure, and without these substances exhibiting potent cytotoxic or other adverse effects in therapeutic applications.

No systems that achieve this are known in the related art, i.e., by which the members of the TNF ligand family are provided in an active and stable form, said members which are suitable for therapeutic application without the aforementioned disadvantages by themselves or as biologically active components of complex fusion proteins.

The object of the present invention is to provide a system by which the stability of the members of the TNF ligand family is increased.

This object is attained by the embodiments of the present invention as characterized in the claims. This object is attained in particular by the subject of claim 1, namely, by the provision of a polypeptide, which comprises at least three components A and at least two components B, whereby each component A is a monomer of a member of the TNF ligand family or a functional fragment, and/or a functional variant thereof and each component B is a peptide linker.

The present invention is based on the knowledge that naturally occurring soluble cytokine members of the TNF ligand family exhibit their full bioactivity only as homotrimers, but, on the other hand, tend to denature via dissociation of their monomers.

It is therefore imperative to prevent this dissociation of the homotrimers into monomers. This is achieved according to the invention in that at least three monomers of a member of the TNF ligand family, particularly TNF, were bound covalently to one another via their C terminals and N terminals by means of short peptide linkers to form a "single-chain" molecule ("sc" hereafter), particularly to form an scTNF. According to the invention, therefore, the entire molecule (at least three monomers of a member of the TNF ligand family with the two peptide linkers) consists of a single protein strand, so that dissociation into monomers can no longer occur. It was demonstrated according to the invention that such molecules of the invention exhibit very low loss of their bioactivity in comparison to their corresponding soluble wild-type members of the TNF ligand family. In contrast, it was not only demonstrated according to the invention that the polypeptides of the present invention have the same (qualitative) activities as their corresponding

soluble wild-type member of the TNF ligand family, but also that because of their considerably higher stability they still exhibit bioactivities at a time when the soluble wild-type member of the TNF ligand family has already lost its activity, i.e., is disassociated or denatured (reference on this point is made to the various stability tests described hereafter, which are described in the examples and in the figures).

A "soluble wild-type member of the TNF ligand family" is to be understood as a soluble extracellular segment of a membrane-associated member of the TNF ligand family. The expressions "wild type", "wt", "soluble," and "s" (for "soluble") are used hereafter synonymously with the term "soluble wild type." These can be in particular soluble wild-type TNF (as a member of the TNF ligand family), for which accordingly the synonymous terms "wild-type TNF," "wtTNF", soluble TNF, and "sTNF" are used hereafter.

A component A within the meaning of the invention is a monomer of a member of the TNF ligand family or a functional fragment or a functional variant thereof. A "monomer" is to be understood as the smallest protein unit or polypeptide unit that can be separated from an oligomeric protein without breaking the covalent bonds.

A polypeptide or a component A or a fragment or a variant thereof is functional within the meaning of the invention, provided it exhibits its biological activity or function, particularly its binding property to an interaction partner, e.g., a membrane-associated receptor, and also its trimerization property. In the case of functional fragments and the functional variants of the invention, these biological functions can in fact be changed, e.g., with respect to their specificity or selectivity, but with retention of the basic biological function.

Numerous methods for measuring the biological activity of a protein, polypeptide, or molecule are known from the related art, for example, protein assays, which use labeled substrates, substrate analyses by chromatographic methods, such as HPLC or thin-layer chromatography, spectrophotometric methods, etc. (see, e.g., Maniatis et al. (2001)

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

A fragment within the meaning of the invention is to be understood as both a fragment of a monomer of a member of the TNF ligand family and a fragment of a polypeptide or protein of the present invention. These can be N-terminally, C-terminally, or intrasequentially shortened amino acid sequences of the monomer, polypeptide, or protein. In particular, the intrasequential shortenings of the polypeptide or protein may be shortenings of the sequence of one or more of the three monomers, which in turn can occur N-terminally, C-terminally, or intrasequentially.

In an especially preferred embodiment of the invention, the fragment of a monomer represents its extracellular domain, which corresponds to the entire extracellular domain of the soluble wild-type member of the TNF ligand family or a segment thereof. In particular, the fragment represents a monomer of its extracellular domain, which corresponds either to the soluble wild-type TNF (amino acids 77-233) or the entire extracellular domain (amino acids 53-233).

The preparation of such fragments of the invention is well known from the related art and can be performed by a person skilled in the art with use of standard methods (see, e.g., Maniatis et al. (2001), Molecular Cloning: Laboratory Manual, Cold Spring Harbor Laboratory Press). In general, the fragments of the monomers, polypeptides, or proteins can be prepared by modification of the DNA sequence, coding for the native monomer, polypeptide, or protein, followed by a transformation of this DNA sequence in a suitable host, and expression of this modified DNA sequence, provided that the modification of the DNA does not disrupt the functional activities of the monomer, polypeptide, or protein.

The identification of a fragment of the invention can occur either by testing its functionality by measuring its biological activity, as described above, or based on a sequencing of the fragment and a subsequent comparison of the obtained sequence with

the native sequence. The sequencing can occur using standard methods, which are well-known and numerous in the related art.

In particular, monomers, polypeptides, or proteins, or fragments thereof that have sequence differences relative to the corresponding native sequences are designated as variants of biologically active monomers, polypeptides, or proteins, or fragments thereof, or a component A within the meaning of the invention. These sequence deviations can be one or more insertion(s), deletion(s), and/or substitution(s) of amino acids, whereby there is a sequence homology of at least 60%, preferably 70%, more preferably 80%, also more preferably 85%, even more preferably 90%, and most preferably 97%.

To determine the percentage identity of two nucleic acid or amino acid sequences, the sequences can be aligned in order to be compared subsequently with one another. To this end, e.g., gaps can be introduced into the sequence of the first amino acid or nucleic acid sequence and the amino acids or nucleotides compared at the corresponding position of the second amino acid or nucleic acid sequence. If a position in the first amino acid sequence is occupied by the same amino acid or the same nucleotide, as is the case at a position in the second sequence, then both sequences are identical at this position. The percentage identity between two sequences is a function of the number of identical positions divided by the sequences.

The determination of the percentage identity of two sequences can be performed using a mathematical algorithm. A preferred, but not limiting example of a mathematical algorithm, which can be used for comparing two sequences, is the algorithm of Karlin et al. (1993), PNAS USA, 90:5873-5877. This type of algorithm is integrated into the NBLAST program, which can identify the sequences that have a desired identity to the sequences of the present invention. To obtain a gapped alignment, as described above, the "gapped BLAST" program can be used, as described in Altschul et al. (1997), Nucleic Acids Res, 25:3389-3402.

Biologically active, therefore functional, variants of monomers, polypeptides, or proteins, or fragments thereof within the meaning of the invention can preferably have selective receptor binding properties, whereby the variant can be optimized, e.g., with respect to their specific bioactivity or other properties, particularly their stability.

The term variants includes particularly amino acid sequences with conservative substitution compared with physiological sequences. Conservative substitutions are substitutions in which amino acids stemming from the same class are exchanged. In particular, there are amino acids with aliphatic side chains, positively or negatively charged side chains, aromatic groups in the side chains, or amino acids whose side chains may contain hydrogen bridges, for example, side chains with a hydroxy function. This means that, for example, an amino acid with a polar side chain is replaced by another amino acid with a likewise polar side chain or, for example, an amino acid characterized by a hydrophobic side chain is substituted by another amino acid with a likewise hydrophobic side chain (e.g., serine (threonine) by threonine (serine) or leucine (isoleucine) by isoleucine (leucine)). Insertions and substitutions are possible particularly at sequence positions that cause no change in the three-dimensional structure or affect the binding region. A change in a three-dimensional structure by insertion(s) or deletion(s) can be easily checked, for example, with the use of CD spectra (circular dichroism spectra) (Urry, 1985, Absorption, Circular Dichroism and ORD of Polypeptides, in: Modern Physical Methods in Biochemistry, Neuberger et al. (eds.), Elsevier, Amsterdam).

Suitable methods for the preparation of variants, e.g., of monomers, polypeptides, or proteins, or fragments thereof with amino acid sequences that have substitutions compared with the native sequences, are disclosed, for example, in the publications U.S. Patent No. 4,737,462, U.S. Patent No. 4,588,585, U.S. Patent No. 4,959,314, U.S. Patent No. 5,116,943, U.S. Patent No. 4,879,111, and U.S. Patent No. 5,017,691. The preparation of variants in general is also described particularly by Maniatis et al, (2001), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press). In this case, codons can be omitted, added, or replaced. Variants can also be particularly proteins or polypeptides that are stabilized to escape physiological degradation, for example, by

stabilization of the protein backbone by substitution of amide-like bonds, for example, also by the use of β-amino acids.

Variants within the meaning of the invention can also be prepared by introducing changes into the nucleic acids, coding for the variants, such as, for example, insertions, deletions, and/or substitutions in one or more nucleotides. Numerous methods for this type of changes in nucleic acid sequences are known in the related art. One of the most frequently used techniques is oligonucleotide-directed, site-specific mutagenesis (see Comack B., Current Protocols in Molecular Biology, 8.01-8.5.9, Ausubel F. et al., 1991 edition). An oligonucleotide whose sequence has a specific mutation is synthesized in this technique. This oligonucleotide is then hybridized with a template that contains the wild-type nucleic acid sequence. A single-stranded template is used preferentially in this technique. After annealing of oligonucleotide and template, a DNA-dependent DNA polymerase is used to synthesize the second strand of the oligonucleotide, which is complementary to the template-DNA strand. A heteroduplex molecule is obtained as a result, which contains a mispairing formed due to the aforementioned mutation in the oligonucleotide. The oligonucleotide sequence is then introduced into a suitable plasmid, this is introduced into a host cell, and the oligonucleotide DNA is replicated in this host cell. This technique produces nucleic acid sequences with selective changes (mutations), which can be used for preparing variants according to the invention.

Components A covered by the polypeptides of the invention can be identical or different; i.e., components A can be monomers of the same member of the TNF ligand family or different members of the TNF ligand family. For example, three different components A can be three monomers of three different members of the TNF ligand family or two monomers of the same member of the TNF ligand family and one monomer of another member of the TNF ligand family. This applies accordingly to more than three components A. For example, a polypeptide of the invention can contain 4, 5, 6, or more components A, which form a tetramer, pentamer, hexamer, etc. It is also especially preferred for the polypeptide of the invention to contain an integer multiple of a trimer, as

previously described, of component A, e.g., two, three, four, or more trimers arranged one after another. Components A present in the polypeptides of the invention can be separated by linkers from one another (see below). If, as described above, two, three, four, or more trimers of components A are arranged one after another, the linkers binding the different trimers to one another can be longer, if necessary, than the linkers binding components A in a single trimer to one another.

Components A of the invention can stem from the same or different organisms. These can be vertebrates, particularly mammals, for example, the mouse, rat, pig, and primarily humans.

In a preferred embodiment of the invention, components A of the polypeptide of the invention are in each case a monomer of one of the members of the TNF ligand family or a functional fragment or a functional variant thereof, selected from the group consisting of FasL (GenBank Accession No. NM_000639), TRAIL (TNF-Related Apoptosis-Inducing Ligand; GenBank Accession No. NM_003810), also called Apo2L, TNF (tumor necrosis factor; GenBank Accession No. NM_000594), LT alpha (GenBank Accession No. NM_000595), lymphotoxin beta (GenBank Accession No. NM 002341), NGF (GenBank Accession No. NM_002506), CD30L (CD153; GenBank Accession No. NM_001244), CD40L (CD154; GenBank Accession No. NM_00074), OX40L (GenBank Accession No. NM_003326), RANKL (GenBank Accession No. NM_003701), TWEAKL (GenBank Accession No. NM_003809), LTalpha, LTbeta, LIGHT (GenBank Accession No. NM_003807), CD27L (GenBank Accession No. NM_001252), 4-1BBL (GenBank Accession No. NM_003811), GITRL (GenBank Accession No. NM_005092), APRIL (GenBank Accession No. NM_172089), EDA (GenBank Accession No. NM_001399), VEGI (GenBank Accession No. NM_005118), and BAFF (GenBank Accession No. NM_006573).

In an especially preferred embodiment, component A, as defined above, of the polypeptide of the invention is a monomer of one of the members of the TNF ligand

family, which is resistant to the processing enzyme TACE. TACE is a member of the ADAM protease family and represents the physiological TNF-specific processing enzyme of TNF found naturally in the cell membrane. TNF is typically cleaved from the cell membrane and released into the environment. A TACE-resistant component A of the invention preferably lacks the Ala-Val cleavage site (e.g., AA 76-77 in scTNF according to the SWISS-PROT nomenclature for human TNF, No. PO1375). The cleavage site can be removed in this case according to the invention by deletion of one or both relevant amino acids Ala or Val. Alternatively or in addition, according to the invention, the recognition sequence for TACE (e.g., AA 77-88 in scTNF according to the SWISS-PROT nomenclature for human TNF, No. PO1375) may be totally or partially deleted. This occurs preferably by deletion of two, three, four, or more amino acids, for example, all amino acids of the TACE recognition sequence. For scTNF, for example, the TACE-resistant sequence is in the region of amino acids 89-233 (AA 89-233) of scTNF according to the SWISS-PROT nomenclature for human TNF, No. PO1375. One such deletion in component A prevents a potential cleavage of component A, e.g., scTNF, by TACE near the linkage sites of components A in the area of the linkers (component B, see below), and thereby increases the *in vivo* stability of the polypeptides of the invention. If necessary, the shortening of the sequence, arising due to the deletion, can be compensated by an appropriate lengthening of the linkers. The deletion of the component A sequence, described as an example for scTNF, can be used for each of the aforementioned components A. If the TACE sequence is not to be removed or to be only partially removed, care must be taken preferably that during use of additional components B or C (see below) in the polypeptides of the invention, the TACE recognition sequence and the TACE cleavage site are not restored.

In another especially preferred embodiment, components A of the polypeptide of the invention, as defined above, are modified in such a way that the polypeptides of the invention can couple covalently to surfaces. This coupling occurs preferably to planar surfaces or to spherical particles, such as, e.g., to magnetic particles (magnetic beads), or to nanoparticles/microparticles, preferably as a cell-mimetic, therapeutic reagent with properties similar to membrane-bound TNF. For coupling, a coupling group, preferably the SH group of a cysteine residue, is introduced in the N-terminal region of at least one

component A of the polypeptide of the invention. (Other preferred coupling groups of the invention are described further below). This can occur especially preferably by the introduction of a cysteine residue in the form of an addition and/or substitution at the desired position. The coupling group can occur at any position of the N-terminal region of component A, preferably near the N terminus. Especially preferably, the coupling group is located in a region of the first 1-15 N-terminal amino acids, and more greatly preferred in a region of the first 1-10 N-terminal amino acids. For example, the modification in a prokaryotically expressed scTNF can be introduced after an initial methionine (position 2) of the amino acid sequence or in a eukaryotically expressed scTNF directly after the cleaved leader sequence; these therefore represent the N-terminal amino acids of component A. Alternatively, in a tag used according to the invention, for example, a His tag, a Flag tag (see, e.g., Fig. 18), a coupling group is introduced according to the invention, e.g., resulting in a CysHis tag with a cysteine at amino acid position 9. Another alternative consists of the introduction of a coupling group in one or both components B (linkers) of the polypeptide of the invention.

Preferably, in a polypeptide of the invention, only the component A situated at the N terminus or the tag situated farther N-terminally is provided/modified with a coupling group as described above. In another embodiment, in the case of a polypeptide of the invention, which contains 3 components A, for example, 2 or 3 of components A modified in each case with a coupling group, so that the entire molecule (the polypeptide of the invention) can be coupled via 2 or 3 covalent bonds to a matrix suitable therefor. The coupling of the polypeptide of the invention via one or more coupling groups of all components A of a polypeptide of the invention preferably does not negatively impact the function of the individual components A, e.g., of TNF, but rather enables improvement of the immobilization, for example, of scTNF, on the surface and/or particle. As previously stated, e.g., in scTNF and also in scFasL and scTRAIL or other members of the TNF family in the sc form, each of polypeptides of the invention contains preferably the subunits necessary for the biological function (components A) in a functionally relevant arrangement. As taught by the invention, it is possible to produce surfaces or particles

with, e.g., coupled TNF homotrimers or heterotrimers or -multimers, by coupling of a polypeptide of the invention to a surface/particle. The coupled TNF homotrimers or heterotrimers or -multimers are immobilized stably and bioactively on these surfaces/particles. The functionally relevant trimer state of components A is stabilized by components B by the single-chain principle of the invention; this assures that after covalent coupling to a surface/particle, dissociation of individual components A is ruled out and therefore loss of biological activity is prevented.

A preferred property of this type of polypeptide coupled to surfaces or particles according to the invention is their biomimetic activity, which corresponds to that of the natural, membrane-associated ligands of the TNF family. For example, an immobilized scTNF of the invention achieves a high affinity for TNFR2 and thereby a high signal capacity.

Polypeptides of the invention, which contain components A modified with a coupling group, as described above, and become/are coupled to a surface and/or particle, as taught by the invention, can be used for detection, as well as for manipulation, depletion, and/or removal of binding partners of members of the TNF ligand family, as well as compounds associated therewith. Of paramount interest thereby are methods for the extracorporeal manipulation, depletion, and/or removal of components present in body fluids, e.g., by apheresis.

The following statements on the subjects of the invention relate particularly to the TNF ligand family member TNF, but they can be applied to all other members of the TNF ligand family.

The polypeptides of the present invention comprise, apart from the described components A (monomers of at least one member of the TNF ligand family), at least two components B, whereby component B has the property of a peptide linker.

Any peptide sequence expressible in a biological system is conceivable as a peptide linker within the meaning of the invention. In the polypeptide constructs of the invention, peptide linkers turned out to be, for example, a flexible compound, which, however, preferably does not negatively influence the intrinsic trimerization properties of the particular member of the TNF ligand family. Preferably, linkers are chosen with properties, particularly flexibility and length, that are capable of stabilizing the spontaneously formed homotrimers of these specific ligands (derivatives).

In a preferred embodiment of the invention, component B accordingly is a peptide linker consisting of 2 to 30, preferably between 4 and 16, and especially preferably between 4 and 12 amino acids, which preferably contain repetitive glycine-serine structures, very preferably Gly-Gly-Gly-Ser modules (GGGS modules) in a repetitive arrangement.

Preferably, the peptide linkers of the invention are therefore peptide linkers rich in glycine (G); i.e., the amino acid sequence of a peptide linker has a high proportion of glycine, preferably from 60 to 80%, especially preferably 75%.

In especially preferred embodiments of the invention, the peptide linker (component B) comprises the amino acid sequence GGGSGGGSGGGS, also called $(GGGS)_3$ or L_{short}, or the amino acid sequence GGGSGGGSGGGSGGS [sic], also called $(GGGS)_4$ or L_{long}. The peptide linkers according to the invention are designated, inter alia, as L1 and L2, so that designations such as $L1_{short}$, $L2_{short}$, $L1_{long}$, and/or $L2_{long}$ result. It is possible to use two different linkers to stabilize a trimer molecule.

Preferably, the peptide linkers according to the invention, therefore components B, in each case link together two of the at least three components A. This linkage occurs covalently via the C terminus of a component A and the N terminus of another component A. According to the invention, the polypeptide represents a single-chain molecule (also called an sc molecule). This means that all components A and components B, which comprise the polypeptide of the invention, are located on a single polypeptide strand or protein strand.

In a preferred embodiment of the invention, components A and components B form a trimeric protein structure. Preferably, this is a homotrimeric protein structure, but the invention also comprises heterotrimeric protein structures.

The formation of this trimeric protein structure is preferably effected by component B and/or enhanced by it. Because component B, which leads to trimerization, or component B, which enhances trimerization, of the polypeptide of the invention substantially should not form any higher aggregates, component B should typically not have any cysteine residues, which can form an intermolecular disulfide bridge. Preferably, component B in a polypeptide of the invention therefore has no cysteine residue or only such cysteine residues, which have an intramolecular disulfide bridge, therefore in the polypeptide itself of the invention, in order to avoid the formation of a covalent bond with the at least one cysteine residue of a fusion protein of another trimer under oxidizing conditions.

The sequences of native polypeptides or fragments of these native polypeptides, which are used as peptide linkers of the invention, can also be present in the form of biologically active functional variants of the same within the meaning of this invention and according to the above definition.

Components B of the invention can be identical or different naturally occurring peptide sequences. They can stem from the same or different organisms. The organisms can be vertebrates, particularly mammals, for example, the mouse, rat, pig, and primarily humans.

The polypeptide of the invention can have additional sequence segments apart from components A and B. Preferred in this regard are so-called tag sequences, for example, at least one Flag tag, therefore the amino acid sequence DYKDDDDK, and/or, for example, at least one His tag (containing several consecutive histidines, for example, at least 5) and/or other tag or antigen sequences. An especially preferred additional sequence

segment is a leader peptide sequence. Preferably, this leader peptide sequence represents a signal for the secretion of the polypeptide or protein in eukaryotic cells. This leader peptide sequence is preferably located N-terminally.

In another embodiment of the invention, a polypeptide of the invention comprises another component C, whereby this component is characterized by a specific interaction with a complementary cell surface molecule ("antigen"). The principle of action of the polypeptide constructs of the invention, such as, for example, constructs of the invention with the apoptosis inducer TRAIL or FasL as component A is particularly applicable for all members of the TNF ligand family that are active as a membrane molecule for specific receptors exclusively or to a particularly good extent. Apart from TRAIL (TNFSF10), FasL (TNFSF6), and TNF (TNFSF2), these include, for example, also the immunomodulators CD40L (TNFSF5) and CD30L (TNFSF8). Component C thereby has a "targeting" function. Accordingly, component C is an antibody fragment, preferably a single-chain antibody fragment, the so-called scFv, or an antibody derivative which recognizes a specific target molecule on the cell surface. In another embodiment, component C is not an antibody-associated protein or peptide, which, in analogy to an antibody-antigen interaction or a receptor-ligand interaction, also selectively recognizes a specific target molecule on the cell surface. In an especially preferred embodiment, the present invention comprises a polypeptide of the invention, as described above, as component A at least one member of the scTNF family, as defined above, e.g., scTNF, and as component C an antibody fragment, as described above, e.g., scFv. Smaller polypeptides of the invention are formed thereby with a "targeting domain" in each case. Such smaller polypeptides of the invention are advantageously less susceptible to, e.g., aggregation.

Preferably, component C is an antigen-binding antibody fragment or an antigen-binding antibody derivative from a mammal, particularly of murine or human origin, or a humanized antibody fragment or a humanized antibody derivative, e.g., of mammalian origin. In the case of derivatization and/or humanizing, component C typically consists of a single-chain Fv derivative, prepared according to the related art, murine component C,

humanized by CDR grafting, or component C is of completely human origin which was transformed accordingly to an scFv derivative.

In another preferred embodiment, component C is a protein ligand or peptide ligand, which as a monomer enters into a specific bond at a membrane receptor.

In another preferred embodiment, component C is a protein or peptide with specificity for cell surface molecules, which is particularly a cytokine receptor, a growth factor receptor, an integrin, or cell adhesion molecule. It is especially preferred that it is a cytokine receptor, which is selected from the group of the TNFR gene family.

Component C of a polypeptide of the invention preferably exhibits specificity for an antigen selectively or predominantly expressed in tumor tissue. This type of tumor antigen can be expressed in general in the malignant cells themselves or also in the nonmalignant part of the tumor, the stromal cells or the tumor endothelium. Such antigens of nonmalignant tissue parts of a solid tumor (carcinoma) are genetically invariant, on the one hand, but on the other, they occur in the most different tumor entities and are thereby universal tumor markers. Examples of such ligands for tumor association, against which a component C of the polypeptide of the invention can be directed, are the VEGFR or the VEGFR/VEGF complex and the integrin $a_v\beta_2$ and the vibronectin isoform β Fn as largely selective target structures of the tumor endothelium and the fibroblast activation protein (as a selective marker of the tumor stroma). All aforementioned examples can be effectively covered by specific scFv, which is why such scFv ("single-chain Fv") are especially suitable as component C of a polypeptide of the invention. Galectin as well is regarded hereby as a tumor marker, against which component C is directed.

Accordingly, antibody fragments in different antibodies formats, for example, scFv, particularly scFv40, are particularly preferred as component C.

In another embodiment of the present invention, the polypeptide via its component C recognizes as a specific target molecule a cell membrane-associated receptor of a member of the TNF ligand family, preferably different from the TNF ligand family member of component A. Examples, which do not represent a definitive enumeration of such possible ligands, are TNFSF1 (LTalpha), TNFSF2 (TNF), TNFSF3 (LTbeta), TNFSF4 (OX40L), TNFSF5 (CD40L), TNFSF6 (FasL), TNFSF7 (CD27L), TNFSF8 (CD30L), TNFSF9 (4-1BBL), TNFSF10 (TRAIL), TNFSF11 (RANKL), TNFSF12 (TWEAKL), TNFSFI3 (APRIL), TNFSF143B (BLYS), TNFSF14 (LIGHT), TNFSFI5 (VEGI), TNFSF16 (CD30L), and TNFSF18 (AITRL), and EDA, which or whose functional fragments or functional variants of the native sequence or the fragments may also be used as component A in a polypeptide construct of the invention. In particular, in this regard, all membrane-associated type II proteins (C terminus extracellular), the functional fragments or functional variants thereof, which cause a trimeric organization of their subunits as a requirement for biological activity, are also disclosed.

Another subject of the present invention is a nucleic acid, which codes for a polypeptide of the invention. This also comprises nucleic acid constructs, which contain a sequence segment coding for a polypeptide of the invention or one or more additional sequence segments. Such additional sequence segments can be, for example, sequences coding for a leader peptide, which is present as a signal for the secretion of the polypeptide or protein according to the invention in eukaryotic cells, or codes for an scFv40, i.e., the sequence of the single-chain (scFv) antibody fragment 40, which is specific for the tumor stroma antigen FAP, or for an "Antibody Mediated Apoptosis Inducing Cytokine" (AMAIZe), or for a HIS/Flag tag, a peptide sequence for the affinity purification of the expressed proteins or polypeptides. This list is provided only as an example and is not definitive.

Other sequences, which may be covered by the nucleic acid sequence, are also noncoding sequences, such as noncoding 3' and 5' sequences, including, e.g., regulatory sequences.

The nucleic acid sequences of the present invention can also be fused with nucleic acid sequences, which, for example, code for a marker sequence or code for a sequence, which codes for a polypeptide, which, for example, facilitates the isolation or purification of the polypeptide of the invention. Representative sequences, for example, include those coding for a glutathione-S transferase (GST) fusion protein, a polyhistidine (e.g., HIS 6), hemagglutinin, or HSV tag. This enumeration is by no means limiting, however.

Nucleic acids of the present invention may be DNA or RNA, particularly mRNA. The nucleic acid molecules can be double- or single-stranded. Single-stranded RNA or DNA can be either the coding (sense) or the noncoding (antisense) strand.

The nucleic acids according to the invention can be present preferably isolated. This means that the nucleic acid molecule for the nucleic acid sequence is not flanked by nucleic acid sequences, which normally flank the gene or the nucleic acid sequence (as in genomic sequences) and/or which were completely or partially purified (such as, for example, in a DNA or RNA library). For example, an isolated nucleic acid of the invention can be isolated in relation to the cellular milieu in which it occurs naturally.

In a preferred embodiment, the nucleic acid comprises one of the nucleic acid sequences presented in **Figs. 19 through 26** or consist of one of these nucleic acid sequences.

According to the invention, functional fragments or functional variants of the nucleic acids or nucleic acid constructs of the invention are also covered, to which the previous statements on the terms functional, fragment, and variant accordingly apply.

The preparation of nucleic acids, nucleic acid constructs, or functional fragments, or functional variants thereof of the invention can be carried out by means of standard methods, which are known to the person skilled in the art (see, e.g., Maniatis et al.

(2001), *supra*). The PCR technique is used particularly in this regard. The sequence of synthesized nucleic acids according to the invention can be determined using sequencing or hybridization methods, which are also familiar to the person skilled in the art.

The invention also comprises gene products of the nucleic acids of the invention. Preferably, the gene product codes for a polypeptide of the amino acid sequences shown in **Figs. 19 through 26**. A gene product of the present invention relates not only to the transcript (mRNA) but also to polypeptides or proteins, preferably in purified form. Alleles, functional fragments, or functional variants of such gene products are also included. The aforementioned definitions of these terms apply accordingly to functional fragments or functional variants of the gene products.

Another subject of the present invention is a vector, which contains the nucleic acid coding for a polypeptide of the invention. Preferably, the vectors of the invention are expression vectors, i.e., vectors that have the ability to express and/or amplify the nucleic acids in a prokaryotic and/or eukaryotic cell. The present invention relates in particular to plasmid vectors, e.g., pBABEpuro, phages, or retroviral vectors, particularly also to all vector systems, which may be used in gene therapy, e.g., also adenoviral vector systems. Within the scope of the present invention, therefore, gene therapy methods with vectors or nucleic acids or nucleic acid constructs of the invention are also disclosed as treatment methods for the disclosed medical indications of the invention.

Vectors of the invention preferably have control sequences, which enable or enhance expression of the nucleic acid of the invention and regulate transcription. Such control sequences include, for example, polyadenylation signals, promoters, e.g., natural or synthetic promoters, enhancers to effect transcription, operator sequences to regulate transcription, silencers for tissue-specific transcription, sequences coding for suitable ribosome binding sites on mRNA, sequences stabilizing mRNA, and sequences

regulating the termination of transcription and/or translation. This represents only an exemplary enumeration of possible control sequences. Other possible control sequences are well known in the related art and are described, for example, by Goeddel (1990), Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA. The control sequences can be modified, e.g., by deletion, addition, or substitution of one or more nucleic acids, to potentiate their control function.

Numerous different promoters for different organisms are known in particular. For example, a preferred promoter for vectors which are used in *Bacillus subtilis* is the AprE promoter; a preferred vector used in *E. coli* is the T7/Lac promoter; a preferred promoter used in *Saccharomyces cerevisiae* is PGK1; a preferred promoter used in *Aspergillus niger* is glaA; and a preferred promoter used in *Trichoderma reesei* (*reesei*) is cbhI. Promoters, which are suitable for use in prokaryotic host cells, include, e.g., beta-lactamase (vector pGX2907 [ATCC39344], which contains the replicon and the beta-lactamase gene), lactose promoter systems (Chang et al. (1978), Nature (London, 275: 615); Goeddel et al. (1979), Nature (London), 281: 544), alkaline phosphatase, the tryptophan (trp) promoter system (vector pATH1 [ATCC37695]), and hybrid promoters, such as the tac promoter (isolated from the plasmid pDR540 [ATCC37282]). However, other bacterial promoters, whose nucleotide sequences are generally known, enable a skilled person to ligate these with a nucleic acid of the invention, whereby linkers or adapters may also be used, to produce the desired restriction sites. Preferably, promoters which are used in bacterial systems also contain a Shine-Dalgarno sequence, which is functionally connected to the nucleic acid.

Suitable expression vectors can consist, for example, of segments of chromosomal, nonchromosomal, and synthetic DNA. Numerous derivatives of SV40 and bacterial plasmids are known for this purpose. Examples are plasmids from *E. coli*, such as colE1, pBK, pCR1, pBR322, pMb9, pUC19, and derivatives thereof, plasmids that can be used in a wide host range, such as RP4, and phage DNA, such as numerous derivatives of the lambda phage, e.g., NM989, and other DNA phages, e.g., M13, and filamentous single-stranded DNA phages, yeast plasmids, vectors, which are suitable for use in eukaryotic

cells, and vectors, which consist of a combination of plasmid and phage DNA. Numerous expression techniques for use of the expression vectors of the invention are known in the related art. Such techniques are, for example, generally described in Maniatis et al. (2001), *supra*.

Another subject of the invention relates to a host cell, which contains the nucleic acid of the invention and/or a vector of the invention.

Host cells within the meaning of the invention are cells that are capable of functioning as host and expression vehicles for a nucleic acid or vector of the invention. These can be prokaryotic and eukaryotic host cells. Prokaryotic - bacterial - host cells are, for example, *Rhodothermus marinus*, *E. coli*, *Streptomyces*, *Pseudomonas, Bacillus, Serratia marcescens*, and *Salmonella thyphimurium*. Eukaryotic host cells comprise, for example, yeasts, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, insect cells, such as Sfp, or mammalian cells, such as COS and CHO. These enumerations are by no means definitive. The selection of a suitable host cell depends on several factors, e.g., in the introduction of a vector into a host cell particularly on the employed vector of the invention.

A further subject of the invention relates to the preparation of a host cell of the invention, which comprises the following steps:

(a) Preparation of a nucleic acid of the invention or a vector of the invention,

(b) Introduction of the nucleic acid and/or vector according to step (a) into a cell.

The preparation of a nucleic acid or a vector can occur as taught by the invention by the already described standard methods and exemplary descriptions. The introduction of the nucleic acid of the invention or the vector of the invention into the host cell can occur with use of any suitable standard method. These include, for example, transformation, electroporation, transfection with the use of, e.g., calcium chloride, lipofection,

infection, transduction, etc. Various standard methods are described, for example, in Maniatis et al. (2001), *supra*.

Another subject of the present invention is a method for preparing a polypeptide of the invention, whereby this method typically comprises the following steps: (a) culturing of a host cell of the invention under suitable conditions, (b) expression of the nucleic acid or nucleic acid construct of the invention under suitable conditions, and (c) isolation of the polypeptide from the host cell and/or culture supernatant.

Culturing a host cell means making it possible for the host cell to grow in a suitable culture medium, at a suitable pH, and at suitable temperatures. Such growth conditions depend on the host cells used in the specific case and are well known to the person skilled in the art. Instructions for culturing cells can also be found in Maniatis et al. (2001), *supra*.

The expression of the polypeptide can occur here typically according to the related art in suitable expression systems, preferably as a secreted product of stable transfectants, e.g., CHO-cells or other animal cells, such as Cos7 or SF9 (insect cells), or other eukaryotic cell systems, for example, *Pichia pastoris*. Preferably, the expressed polypeptides of the invention each have leader sequences suitable for secretion in the cell system. For this reason, the vectors used for expression according to the invention also contain coding segments, which code for a functional leader sequence, e.g., as described in Brocks et al. (Immunotechnology 3:173-184, 1997) for mammals and insect cells or during use of, for example, pPIC-Zalpha vectors (Invitrogen, Karlsruhe, Germany) for expression and secretion in the yeast *Pichia pastoris*.

The isolation of the polypeptide of the invention from the host cell can occur with use of standard methods, such as chromatography methods, precipitation methods, etc., which are suitable for purification of polypeptides and proteins (also see Maniatis et al. (2001), *supra*).

In another embodiment of the invention, the polypeptides, but optionally also nucleic acids, nucleic acid constructs, vectors, or host cells (combined here in the category of "substances of the invention") of the invention can also be used as a medication or for the preparation of medications for the treatment of diseases.

Provided these substances of the invention comprise a component C according to the invention (see above, an antibody fragment or another specifically binding protein/peptide), their use is appropriate particularly if the substances after binding of the polypeptide or fusion protein to a specific cell membrane-expressed target molecule exhibit the full biological effect via the appropriate receptor of the member of the TNF ligand family. By suitable selection of the specificity of the targeting component C, the activity of the substance of the invention is directed to the tissue to be treated, e.g., tumor tissue, and a therapeutic agent specifically optimized/intended for the particular indication/tumor entity can be prepared. A polypeptide of the invention is accumulated, e.g., during use as a tumor therapeutic agent, particularly for the treatment of solid tumors, but also of lymphatic tumors (benign or malignant), after in vivo administration by the targeting component C initially specifically in the tumor region by membrane markers formed by the tumor itself or the reactive tumor stroma/tumor vascular system and there presented to TNF-receptor-family-positive tumor cells or sensitive cells of the reactive tumor-supplying normal tissue, sensitive to a member of the TNF ligand family.

The use of substances of the invention, however, is always basically desirable for application in the therapeutic area, when the activation of a signal transduction chain, e.g., the signal cascades triggered by the TNF receptor family, for example, an apoptotic signal cascade, is to be triggered. Thus, the substances of the invention are used in the treatment or for the preparation of a medication for the treatment of all hyperproliferative diseases, for example, also for the targeted elimination of cells of the immune system in excessive immune responses, for example, in autoimmune diseases, such as, e.g., multiple sclerosis, rheumatoid arthritis, diabetes mellitus, and TEN, or in misdirected immune responses to foreign antigens, as can occur, e.g., in infectious diseases (bacterial (for example, caused by mycobacteria), viral, or protozoan). Also possible furthermore is

the treatment of metabolic diseases or general hyperinflammatory conditions, particularly chronic inflammations, for example, also in allergies but also the treatment of rejection reactions of a patient's immune system to foreign tissue. In the aforementioned cases, the polypeptide of the invention must recognize, e.g., via a component C, a characteristic marker on the surface of the target cells, in which preferably an apoptotic signal cascade is to be triggered with the goal of cell death. In the case of treatment after transplantation of foreign tissue, therefore, for example, the body's own cells responsible for the rejection reaction in the transplantation patient's immune system serve as the target cells.

In general, the substances of the invention or medications are suitable for the treatment of cancer, particularly solid or lymphatic tumors, infectious diseases, metabolic diseases, inflammatory conditions, hyperproliferative diseases, autoimmune diseases, particularly rheumatoid/arthritic diseases, toxic epidermal necrolysis (TEN), multiple sclerosis, Hashimoto's thyroiditis, GVHD, viral hepatitis (HBV, HCV), alcohol-induced hepatitis, rejection reactions in liver transplantation, diseases based on hyperapoptotic reactions, and degenerative diseases, particularly neurodegenerative diseases.

Recombinant protein is preferably administered to the patient to be treated during use of the substances of the invention as a medication for the treatment of the aforementioned diseases. Alternatively, cells are removed from the patient for transfection; these are transfected *in vitro* with the (expression) vectors of the invention, cultured, and then given to the patient as a retransplant. The transfection is preferably performed with nucleic acids, nucleic acid constructs, or (expression) vectors, which couple the expression to a regulatable promoter. The transfected autotransplant, for example, can be injected locally, depending on the specific disease and the specific target cells. Local administration is preferred, for example, in the case of tumor therapy. In this case, tumor cells are removed from the patient, transfected *in vitro*, and then, provided this is possible, injected directly into the tumor, for example, for the treatment of skin tumors (e.g., melanomas), and tumors of the nervous system (e.g., glioblastomas).

Other subjects of the present invention relate to the use of the substances of the invention both for the preparation of a vaccine for active or passive immunization against infectious diseases, particularly against viral infectious diseases, and also for the preparation of a vaccine for vaccination against German measles, measles, poliomyelitis, rabies, tetanus, diphtheria, BCG, malaria, yellow fever, HIV, or influenza.

Substances of the invention can also be used for *in vitro* diagnosis.

Another subject of the present invention relates to methods for extracorporeal (*ex vivo*) manipulation, depletion, and/or removal of components present in body fluids, such as, e.g., binding partners of a component A, as defined above, or cells binding thereto or associated therewith. Such extracorporeal methods comprise preferably methods such as, e.g., apheresis, particularly the basic forms of apheresis, plasmapheresis and cytapheresis.

Plasmapheresis according to the invention includes extracorporeal manipulation, depletion, and/or removal of certain soluble or suspended components in the plasma fraction of blood, and the return of the thus treated blood to the patient. To do this, peripheral blood is removed from a patient preferably by means of a pheresis machine; anticoagulant agents are optionally added to the blood, and the blood is separated into its major components—solid (red blood cells, white blood cells, and platelets) and liquid fractions (plasma). After separation into these main components, the soluble or suspended blood components, present in the thus obtained plasma fraction, can be manipulated, depleted, and/or removed in another process step, for example, with use of the polypeptides of the invention. Next, the thus treated blood plasma together with the previously separated solid blood components can be combined and reinjected into the patient. The volume loss due to the plasmapheresis can be later replaced by isotonic saline solution in the method of the invention. Plasmapheresis as taught by the invention is preferably carried out with the use of methods such as therapeutic plasma exchange (TPE), immunabsorption (IA), precipitation (HELP), differential membrane filtration, and other means. Plasma filtration columns are mentioned here as examples (see, e.g.,

U.S. Patent No. 4,619,639, Asahi Medical Company, incorporated here by reference). Membrane filtration systems (MDF) with use of different particles and surfaces, for example, filters such as PlasmaFlo®OP-05(W)L and RheoFilter®AR2000 blood filters, can be used (manufactured by Asahi Medical Company, Ltd. of Japan). Alternatively, all suitable surfaces and particles can be used in the plasmapheresis methods of the invention.

In cytapheresis, as taught by the invention, in contrast to the previously described plasmapheresis, cellular components circulating in the blood and/or bound to bone marrow (red blood cells, white blood cells, stem cells, or platelets) or specific subpopulations of these cells are manipulated, depleted, and/or removed extracorporeally to achieve a clinical effect. Here as well, peripheral blood is taken from a patient by means of a pheresis machine. Anticoagulant agents are optionally then added to the blood and the blood is separated into its main components. Next, in the blood fraction containing the cell components, these cell components can be manipulated, depleted, and/or removed in another process step, preferably also with use of the polypeptides of the invention. In the end, the thus treated fraction can be reinjected into the patient. Preferably, in the cytapheresis of the invention, the separation of the blood into various fractions and optionally the separation of certain cell components in blood occur using methods such as centrifugation, differential membrane filtration, or other means. Furthermore, membrane filtration systems (MDF) with use of different particles and surfaces, for example, filters such as PlasmaFlo®OP-05(W)L and RheoFilter®AR2000 blood filters, can be used (manufactured by Asahi Medical Company, Ltd. of Japan).

In a third alternative of the invention, the aforementioned methods of plasmapheresis and cytapheresis can be combined, for example, to manipulate, deplete, and/or remove both soluble or suspended components in the plasma fraction of blood and cellular components circulating in blood and/or bound to bone marrow (red blood cells, white

blood cells, stem cells, or platelets) or specific subpopulations of these cells in order to achieve a clinical effect. Such a combination of plasmapheresis and cytapheresis is preferably produced with use of the polypeptides of the invention.

In a preferred embodiment, the present invention relates to an (apheresis) method for extracorporeal manipulation, depletion, and/or removal of soluble, suspended, or cellular blood components comprising the following steps:

- Optionally separation of the blood into one or more fractions with solid and/or liquid components;
- Binding of soluble, suspended, or cellular blood components to a surface or particle coupled to a polypeptide of the invention; and
- Optionally separation of the bound soluble, suspended, or cellular blood components.

In a special embodiment of the apheresis method of the invention, blood can be optionally removed from a patient before the separation of the blood if necessary. Furthermore, the blood treated by the method of the invention or the thus treated blood fraction can be reinjected into the patient. To do this, it may be necessary to recombine the previously separated and possibly differently treated blood fractions with other untreated solid and/or liquid components of blood. The volume loss due to the method of the invention can be compensated by addition of appropriate fluids, e.g., by addition of an isotonic saline solution.

The step in the apheresis method of the invention for binding the soluble, suspended, or cellular blood components to a surface or particle coupled to a polypeptide of the invention can be carried out once or repeatedly as needed, in order to achieve a desired selectivity.

In the apheresis method of the invention, such surfaces and/or particles are used which were covalently coupled to the polypeptide of the invention, as defined above. To this

end, the polypeptide of the invention is preferably covalently coupled to the surface and/or the particle via the coupling group(s) A present in the polypeptide.

Coupling of the polypeptides used according to the invention via the individual components A occurs preferably as described in DE 101 44 252 A1. The coupling of the polypeptides used according to the invention occurs thereby preferably to the surfaces or particles (carriers) via a bond between the first functional groups present on the carrier surface and in the polypeptide via the coupling groups present in components A. These coupling groups are preferably complementary to the functional groups of the carrier and can enter into affinity, preferably covalent bonds with these. Preferably, the functional group of component A is positioned within component A such that it is placed a suitable distance outside the domains, responsible for the biological activity, of the polypeptide of the invention. In this way, it is possible according to the invention to immobilize TNF directionally on the carrier and with retention of its biological activity. After the immobilization, the polypeptide of the invention is fixed preferably to the carrier's surface such that the three-dimensional structure of the domain(s) necessary for biological activity has not been altered compared with the non-immobilized polypeptide of the invention, and that the domain(s) during contact with cellular reaction partners is/are freely accessible for these.

In a preferred embodiment of the invention, the functional group of the carrier surface is selected from the group consisting of the amino group, carboxy group, epoxy group, maleimido group, alkylketone group, aldehyde group, hydrazine group, hydrazide group, thiol group, and thioester group.

According to the invention, the coupling groups of components A, to be immobilized, of the polypeptide of the invention are selected from a group, which contains the same species as for the functional group of the carrier surface. A surface/particle (= carrier), which can be used in the apheresis method of the invention, therefore, has on its surface a functional group, which is covalently linked to a coupling group of the polypeptide, to be immobilized, of the invention, whereby the coupling group of the polypeptide is a group

different from the functional protein of the carrier. The two coupling groups and functional groups, binding together, must thereby be complementary to one another, that is, be capable of entering into a covalent bond with one another. If, for example, an amino group is used as the functional group of the carrier according to the invention, the coupling group of component A of the polypeptide of the invention is a carboxy group. If conversely a carboxy group is used as the functional group of the carrier according to the invention, the coupling group of component A of the polypeptide of the invention is an amino group as taught by the invention. If a thiol group is selected as the functional group of the carrier according to the invention, the complementary coupling group of component A of the polypeptide of the invention is a maleimido group as taught by the invention. If conversely a maleimido group is used as the functional group of the carrier, the complementary coupling group of component A of the polypeptide of the invention is a thiol group as taught by the invention. If an alkylketone group, particularly a methylketone or aldehyde group, is used as the functional group of the carrier according to the invention, the functional complementary coupling group of component A of the polypeptide of the invention is a hydrazine or hydrazide group. If conversely according to the invention a hydrazine or hydrazide group is used as the functional group of the carrier, the functional complementary coupling group of component A of the polypeptide of the invention is an alkylketone, particularly methylketone or aldehyde group, as taught by the invention. According to the invention, the functional group on the carrier surface is preferably a maleimido group, and in the coupling group of component A of the polypeptide of the invention, a thiol group.

The immobilization thereby occurs preferably directionally. In regard to the present invention, the term "immobilized directionally" or "directional immobilization" means that a polypeptide of the invention, particularly an scTNF member, is immobilized at a defined position within the scTNF member in such a way on a carrier that the three-dimensional structure of the domain(s) necessary for biological activity has not changed compared with the non-immobilized state and that this (these) TNF domain(s), for example, binding pockets for cellular reaction partners, during contact with cellular reaction partners is/are really accessible for these. "Immobilized directionally" also means that the coupling of the polypeptide of the invention on the carrier surface occurs

in such a way that the immobilized protein during later use in a cellular and/or cell-like environment cannot be degraded or degraded only very slowly by protein-degrading enzymes. This means that an immobilized TNF molecule of the invention is aligned on the carrier surface in such a way that it offers as few sites of action as possible for proteases. In addition, the polypeptide of the invention before coupling can be made resistant to proteases by biomolecular methods, as described above, for example, for TNF members in the case of protease TACE.

The polypeptide of the invention via its coupled component(s) A retains its biological function and the ability to interact with other compounds/substances. If a polypeptide of the invention contains, for example, three components A (trimer), then typically each, two, or preferably only one of the three components A are bound covalently to the surface/particle.

For coupling of suitable surfaces and/or particles, all suitable surfaces and/or particles can be included to which the polypeptides of the invention can be coupled. In particular, culture plates or so-called microbeads, e.g., Dynabeads (Dynal Biotech GMBH), nanobeads, nanoparticles, or solid phases such as, for example, nylon wool, Sepharose, Sephadex, etc., are especially preferred. According to the invention, it is provided furthermore that the carrier systems used according to the invention are compact or hollow nanoparticles between 25 and 1000 nm in size. These consist either of organic or inorganic particle materials. The type of the material can be varied as taught by the invention according to the subsequent use, whereby the carrier of the nanoparticles consists preferably of biologically compatible and/or biologically degradable materials. In regard to the present invention, "nanoparticles" are understood to be furthermore binding matrixes, which comprise a carrier with a surface on which chemically reactive functional groups are arranged, which enter into affinity bonds, thus covalent and/or noncovalent bonds, with complementary functional groups of molecules to be bound, particularly the polypeptides of the invention, and in this way can stably fix the polypeptides of the invention on their surface. The carriers of the nanoparticles consist of chemically inert inorganic or organic materials and are less than 1 μm in size, preferably from 25 to 500 nm.

A special embodiment of the apheresis method of the invention comprises a biofunctionalized surface (planar or as particles), e.g., with the polypeptide of the invention scTNF, which was immobilized covalently and directionally on the surface in the previously described manner. This type of biofunctionalized surface, containing, for example, several immobilized scTNF molecules or variants thereof, makes possible a high affinity for the specific complementary binding partner, for example, TNFR1 and TNFR2. The receptors TNFR1 and particularly TNFR2 are found in highly elevated concentrations as processed, soluble molecules in the blood of tumor patients and in other diseases. The removal of TNFR from the blood of tumor patients demonstrably leads to clinically documented tumor regression, which is attributed to a reconstitution of the body's own defense (see, for example, Lentz M.R., (1999) Therapeutics Apheresis, Vol. 3, No. 1). An apheresis method with polypeptides of the invention, for example, scTNF, makes it possible that preferably both TNF receptors can be removed simultaneously and efficiently from samples. Another advantage of this method is the above-described fact that dissociation of individual components A of the polypeptide of the invention is not possible; i.e., neither an activity loss of the functionalized surface nor contamination of the treated blood with the associated substances is expected.

The apheresis method of the invention can be carried out as a continuous or discontinuous process. As a continuous process, the method of the invention is preferably carried out directly after the taking of blood or the fractionation of the removed blood and before the return of the blood to the patient without interim storage of the blood or the obtained blood fractions. In a discontinuous process, in contrast, the removed blood or the fractions of the removed blood can be stored after each step for a specific time period.

The temperature in the apheresis method of the invention is preferably 0 to 40 °C. In a continuous process, the temperature is preferably 25 to 40 °C, especially preferably in a

range between 36 °C and 38 °C. In a discontinuous process, the temperature can be between 0 °C and 40 °C.

The apheresis method of the invention as described above is used preferably for diagnosis, therapy, and/or prophylaxis in diseases associated with members of the TNF ligand family, particularly in tumor diseases, particularly solid or lymphatic tumors, particularly solid and lymphatic tumors [sic]. The restoration of the homeostasis of the immune system with its humoral and cellular components is regarded as the key to the effectiveness of the apheresis method. In another application, the apheresis system of the invention can be used for this reason also in restoring the immune homeostasis in nonmalignant diseases. These include inflammatory diseases, arthritic and rheumatic diseases, or diseases of the immune system, as well as for the treatment [sic] of infectious diseases, metabolic diseases, inflammatory conditions, hyperproliferative diseases, autoimmune diseases, particularly rheumatoid/arthritic diseases, toxic epidermal necrolysis (TEN), multiple sclerosis, Hashimoto's thyroiditis, GVHD, viral hepatitis (HBV, HCV), alcohol-induced hepatitis, rejection reactions in liver transplantation, diseases based on hyperapoptotic reactions, and degenerative diseases, particularly neurodegenerative diseases.

Tumor diseases comprise in particular colon cancer, melanomas, renal carcinomas, lymphomas, acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL), gastrointestinal tumors, lung carcinomas, gliomas, thyroid tumors, breast carcinomas, prostate tumors, hepatomas, various virus-induced tumors, such as, e.g., papilloma virus-induced cancer (e.g., cervical carcinoma), adenocarcinomas, herpes virus-induced tumors (e.g., Burkitt's lymphoma, EBV-induced B cell lymphoma), hepatitis B-induced tumors (hepatocellular carcinomas), HTLV-1 and HTLV-2 induced lymphomas, acoustic neurinoma, cervical cancer, lung cancer, throat cancer, anal carcinoma, glioblastoma, lymphomas, rectal cancer, astrocytoma, brain tumors, stomach cancer, retinoblastoma, basalioma, brain metastases, medulloblastomas, vaginal cancer, pancreatic cancer, testicular cancer, melanoma, thyroid cancer, bladder cancer, Hodgkin's syndrome, meningeomas,

Schneeberg's disease, bronchogenic carcinoma, pituitary tumor, mycosis fungoides, esophageal cancer, breast cancer, carcinoids, neurinoma, spinocellular carcinoma, Burkitt's lymphoma, laryngeal cancer, kidney cancer, thymoma, uterine carcinoma, bone cancer, non-Hodgkin's lymphoma, urethral cancer, CUP syndrome, head-neck tumors, oligodendroglioma, vulvar cancer, intestinal cancer, colon cancer [sic], esophageal carcinoma, wart involvement, small bowel tumors, craniopharyngeomas, ovarian carcinoma, soft tissue tumors, ovarian cancer, liver cancer, pancreatic carcinoma, cervical carcinoma, endometrial carcinoma, liver metastases, penile cancer, tongue cancer, gallbladder cancer, leukemia, plasmocytoma, uterine cancer, lid tumor, prostate cancer, etc.

Arthritic diseases comprise particularly monarthritis, oligoarthritis, polyarthritis, acute arthritis (primarily septic, crystal-induced, reactive arthritis and acute arcoidosis), subacute arthritis, chronic arthritis (primarily rheumatoid arthritis, arthritis in seronegative spondylarthritis), infectious arthritis, para- or postinfectious arthritis, rheumatoid arthritis, juvenile chronic arthritis, arthritis in inflammatory connective tissue diseases and vasculitis, allergic arthritis, arthritis in conjunction with metabolic diseases and nutrition-induced disorders, arthritis in endocrine disturbances, arthritis in granulomatous diseases, arthritis in diseases of the hematopoietic system, arthritis in joint hemorrhaging due in blood coagulation disorders, neoplastic arthritis, paraneoplastic arthritis, (post-)traumatic arthritis, arthritis in diseases of the articular cartilage, arthritis in neuropathies, arthritis in pustular, abscessing, necrotizing dermatoses or dermatoses proceeding with tissue neutrophilia, arthritis in other extra-articular primary diseases, as well as allergic arthritis, chlamydia-induced arthritis, dysenteric arthritis, gonorrheal arthritis, arthritis mutilans, psoriatic arthritis, dry arthritis, syphilitic arthritis, tuberculous arthritis, uric arthritis, etc.

Another subject of the present invention is a pharmaceutical composition or a vaccine, containing the polypeptides of the invention, nucleic acid constructs of the invention, vectors of the invention, and/or host cells of the invention, as well as pharmaceutically compatible aids, additives, and/or carrier substances (e.g., also solubilizers). The

pharmaceutical compositions or vaccines according to the invention are preferably used for the treatment of cancer diseases, particularly solid or lymphatic tumors, as well as for the treatment of infectious diseases, metabolic diseases, inflammatory conditions, hyperproliferative diseases, autoimmune diseases, particularly rheumatoid/arthritic diseases, toxic epidermal necrolysis (TEN), multiple sclerosis, Hashimoto's thyroiditis, GVHD, viral hepatitis (HBV, HCV), alcohol-induced hepatitis, rejection reactions in liver transplantation, diseases based on hyperapoptotic reactions, and degenerative diseases, particularly neurodegenerative diseases.

Therefore, according to the invention, a combination of substances of the invention with pharmaceutically acceptable carriers, aids, and/or additives is also disclosed. Appropriate production methods are disclosed in "Remington's Pharmaceutical Sciences" (Mack Pub. Co., Easton, PA, 1980), which is part of the disclosure of the present invention. For parenteral administration, for example, sterile water, sterile saline solution, polyalkylene glycols, hydrogenated naphthalene, and particularly biocompatible lactide polymers, lactide/glycolide copolymers, or polyoxyethylene/polyoxypropylene copolymers may be considered as carrier materials. Pharmaceutical compositions of this type according to the invention may be used for all medical indications disclosed above. In addition, the compositions of the invention may contain fillers or substances, such as lactose, mannitol, substances for the covalent linking of polymers, such as, e.g., polyethylene glycol to inhibitors of the invention, complexing with metal ions or inclusion of materials in or on special preparations of polymer compounds, such as, e.g., polylactate, polyglycolic acid, hydrogel, or on liposomes, microemulsion, micelles, unilamellar or multilamellar vesicles, erythrocyte fragments, or spheroplasts. The specific embodiments of the compositions are selected depending on the physical behavior, for example, in regard to solubility, stability, bioavailability, or degradability. Controlled or constant release of the active components of the invention in the composition includes formulations based on lipophilic depots (e.g., fatty acids, waxes, or oils). Coatings of substances of the invention or compositions containing such substances, namely, coatings with polymers, are disclosed within the scope of the present invention (e.g., poloxamers

or poloxamines). Furthermore, the substances or compositions of the invention may have protective coatings, e.g., protease inhibitors or permeability enhancers.

All administration routes known in the related art are disclosed basically within the scope of the present invention for the substances of the invention or the pharmaceutical compositions of the invention or vaccines; preferably the manufacture of a medication or a vaccine occurs for the treatment of the aforementioned diseases or disorders by the parenteral, i.e., for example, subcutaneous, intramuscular, or intravenous, oral, intranasal, intra-aural, transdermal, topical (e.g., via gels, ointments, lotions, creams, etc.), intraperitoneal, intrapulmonary (e.g., AERx® inhalation technology, commercially available from Aradigm or Inhance™ pulmonary delivery system, commercially available from Inhale Therapeutics), vaginal, rectal, or intraocular administration routes. Typically, pharmaceutical compositions of the invention are solid, liquid, or aerosol-like (e.g., spray), depending on the packaging or administration.

In a preferred embodiment, the therapeutically effective amount of a polypeptide of the invention is administered as needed to a patient. The precise dose typically depends on the purpose of the basic treatment and can be determined by a person skilled in the art with use of known methods from the related art. As is known from the related art, adjustments may be necessary, for example, in regard to the metabolic breakdown of the polypeptides of the invention, in regard to systemic versus local administration, as well as to the age, body weight, general state of health, gender, nutrition, time of administration, interaction of active ingredients, or the severity of the disease. Such adjustments can be made by a person skilled in the art by means of methods known in the related art.

In a preferred embodiment, a combination of polypeptides of the invention for use as adjuvant is disclosed. An adjuvant within the meaning of this invention is particularly a composition that causes no specific immune response to an immunogen, but is capable of enhancing the immune response to this immunogen. Stated differently, administration of

the adjuvant alone causes no immune response to this immunogen, whereas administration together with this immunogen produces an immune response, which is greater than the immune response after administration of the immunogen alone. An adjuvant can contain in addition pharmaceutically acceptable carriers, aids, and/or additives or be administered with them, as is disclosed here for vaccines and pharmaceutical compositions. Furthermore, an adjuvant can be administered together with the vaccines or pharmaceutical compositions disclosed above.

It can be stated in summary that the invention provides a polypeptide, which has increased stability, as a result of which the entire molecule of a member of the TNF ligand family consists of a protein strand or polypeptide strand (e.g., scTNF), so that the monomers (components A) of the members of the TNF ligand family can no longer dissociate. scTNF, as well as the polypeptides of the invention, which relate to other members of the TNF ligand family, exhibits no qualitative differences in its bioactivity to the normal soluble sTNF (also wild-type TNF or wtTNF), but is more stable by far and therefore exhibits higher bioactivity, and thus is effective at lower concentrations. Like the normally soluble TNF (wtTNF), scTNF was able to trigger apoptosis in sensitive cells, to activate the transcription factor NF-κB, the receptor TNFR1 but not TNFR2, and in the presence of certain antibodies (e.g., 80M2) also to activate the receptor TNFR2. This applies to polypeptides of the invention, which relate to other members of the TNF ligand family. When the polypeptides of the invention are used with a coupling group to produce biofunctionalized surfaces and the use thereof, for example, for apheresis, no bleeding of the polypeptide of the invention or a return of harmful ligands into the system is observed.

Furthermore, the polypeptide of the invention, for example, scTNF, represents an ideal starting material for the preparation of new bifunctional molecules. On the one hand, the polypeptide of the invention, for example, scTNF, or functional fragments thereof can bind covalently to cell surfaces. According to the invention, this has the advantage that only a single covalent bond must be created for stable binding of the molecule. In a normal soluble wild-type member of the TNF ligand family, for example, soluble sTNF, all three monomers of the individual homotrimer must be covalently linked to the surface

in order to obtain a stable construction, because otherwise the non-covalently bound monomers dissociate off. In this embodiment of the polypeptide of the invention, use in apheresis, e.g., with scTNF as a biofunctional active substance to remove TNF receptors from body fluids is especially advantageous.

This also applies to the preparation of fusion proteins. These are especially interesting in order to concentrate a member of the TNF ligand family, for example, TNF, with use of an antibody fragment fused to TNF, for example, scFv, at a specific desired location in the body (so-called "targeting" strategies). In normal soluble TNF (wtTNF), this type of fusion protein must consist of homotrimeric TNF, in which each of the at least three monomers carries the antibody moiety, as a result of which large and unstable molecules form, which in turn dissociate into their monomers and/or tend to aggregate and thereby can be inactivated. A fusion protein derived from scTNF is linked with only one molecule of the antibody, so that the entire fusion protein in turn consists of a single, stable protein strand or polypeptide strand. The descriptions for TNF above can be applied to all members of the TNF ligand family.

It is also possible by means of the present invention, due to the covalent linkage of the monomers (components A) of the polypeptide of the invention, to introduce selectively and stably mutations in only one or also two or more of the at least three monomers (components A). Thus, e.g., point mutations are known, which force receptor selectivity; i.e., for example, in the case of TNF, binding still occurs only at one of the two TNF receptors (TNFR1 and TNFR2). It is possible according to the invention to produce for the first time, e.g., TNF mutants, which are capable of selectively binding a molecule of TNFR1 and two molecules TNFR2 simultaneously; i.e., heteromeric receptor complexes would result. In this way it is possible for the first time to prepare heteromeric, stable, single-chain members of the TNF ligand family, which selectively bind and activate only one of several possible receptors.

Of course, the explanatory statements made above as examples in regard to TNF can be applied to the other members of the TNF ligand family.

The present invention will be illustrated in greater detail by means of the following figures:

Figures 1-3 show the results of different biological cytotoxicity tests, in which the properties, particularly the specificity, of different scTNF variants were compared with those of classic soluble wild-type TNF, i.e., the soluble extracellular domain of human TNF (NCBI gi:25952111, 17.09 kDa, as a monomer of the soluble form, abbreviated TNFhum or sTNF, AA 79-181).

Both transfected mouse fibroblasts and human Kym1 cells were used for these tests. Exemplary results of the analyses are presented, whereby scTNF variants were tested with peptide linkers of different length between the individual TNF modules (i.e., TNF monomers). The peptide linkers in each case consist of the threefold or fourfold amino acid repetitions GGGS (or also GlyGlyGly-Ser) (designated as "single-chain TNF3x" or "single-chain TNF4x" or as "scTNF3x" or "scTNF4x").

Figure 1 shows the result of a typical cytotoxicity test of TNF. Mouse fibroblasts (MF) from TNFR1/TNFR2 double-knockout mice, which stably express TNFR1 receptor chimeras (TNFR1-Fas) (MF TNFR1-Fas cells) were used as target cells. Such cells express a hybrid molecule, which consists extracellularly of the corresponding part of TNFR1 and thereby binds TNF, and intracellularly has the highly apoptotically acting intracellular domain of the Fas-receptor. These MF TNFR1-Fas cells can be activated by TNFR1-specific stimuli, but mediate a Fas-specific death signal. The surviving cell count was quantified after 24 hours by absorption with staining with a dye. A control bacterial lysate was used as a negative control, which was a non-scTNF expressing bacterial lysate, which was processed identically to the recombinant scTNF proteins. It can be stated as a result that both the conventional human soluble TNF (TNFhum) and the recombinant scTNF variants led to cell death. A comparable dose-dependent cytotoxic effect of TNFhum or scTNF is evident (see the bottom three curves in the diagram), whereby the

specific activity of the scTNF is at least equivalent, if not even higher, than that of the wild-type TNF. As expected, the negative control shows no toxic effect.

In another test batch, the effect of neutralizing TNF-specific antibodies on the cytotoxicity of wild-type TNF (TNFhum) or scTNF was tested. Freshly prepared dilutions of soluble human TNF (TNFhum) or the above-described scTNF variants with threefold (3x) or fourfold (4x) glycine-serine peptide linkers (scTNF3x: GGGS-GGGS-GGGS / scTNF4x: GGGS-GGGS-GGGS-GGGS) were used. Concentration series with 1:3 dilutions in the presence and absence of neutralizing TNF-specific antibodies (αTNF-AB) [1 µg/mL] were used. The above-described control bacterial lysate was used as the negative control. It is evident as the result that the cytotoxic effect of soluble human wild-type TNF or scTNF can be abolished by neutralizing TNF-specific antibodies (see the top three curves in the diagram), namely, within a broad concentration range (0.03-10 ng/mL). No differences were found between the reaction of scTNF with 3x and scTNF with 4x peptide linkers.

Figure 2 shows the result of a cytotoxicity test in which the effect of sTNF and scTNF variants on TNFR2-receptor chimera (TNFR2-Fas) expressing cells was studied. In this test batch, mouse fibroblasts were used which stably express receptor chimeras consisting of TNFR2 and Fas (TNFR2-Fas, whereby the extracellular part stems from TNFR2 and the intracellular part from the Fas-receptor) (MF TNFR2-Fas cells). MF TNFR2-Fas cells (like the wild-type TNFR2) can only be activated by an adequate TNFR2 stimulus, for example, by membrane-associated TNF, but not by soluble TNF. Result: As expected, both the soluble human TNF (TNFhuman) and the scTNF variants with the 3x and 4x peptide linkers (scTNF3x, scTNF4x) had no toxic effect on the MF TNFR2-Fas cells. In other words, sTNF and the scTNF variants are equally incapable of activating TNFR2-chimera (TNFR2-Fas) expressing cells; i.e., they cause no cell death in these cells. The control bacterial lysate used as the negative control (as described above) as expected also had no toxic effect.

Figure 3 shows the result of a cytotoxicity test in which the effect of soluble human wild-type TNF and an scTNF variant in combination with a special TNFR2-specific antibody, 80M2, on TNFR2-chimera (TNFR2-Fas) expressing cells was studied. The test batch corresponds that from Fig. 2 with the difference that the soluble wild-type TNF or the scTNF with 3x peptide linkers (scTNF3x) was given together with the antibody 80M2. The antibody 80M2 is known for conferring the special signal capacity of membrane-bound TNF on normal soluble TNF. The result shows that both the soluble wild-type TNF and scTNF in combination with the antibody 80M2 achieve a strong cytotoxic effect via the TNFR2-Fas receptor chimeras. The reason for this is that soluble TNF in combination with certain TNFR2-specific antibodies, such as, e.g., 80M2, can exhibit the activity of membrane-bound TNF. MF TNFR2-Fas cells die after incubation with soluble TNF or scTNF in the presence of such a ligand-receptor complex-stabilizing antibody (80M2-AB). In other words, in the presence of this antibody, soluble TNF and scTNF have a similar toxic effect in the MF TNFR2/FAS cells, as this otherwise can occur only with membrane-associated TNF. The control bacterial lysate used as the negative control (as described above in Fig. 1) as expected had no toxic effect.

Figures 4-10 show the results of different stability tests, which demonstrate that the scTNF variants of the invention due to their structure have a vastly better stability than soluble wild-type TNF.

sTNF or the scTNF variants were incubated in a serum-containing culture medium at concentrations of 3.0 to 0.01 ng/mL at 37 °C and 5% CO_2 for different time periods. After this, the functionality of the sTNF or scTNF variants (scTNF3x, scTNF4x) was tested in the cytotoxicity tests based on MF TNFR1-Fas cells and Kym1 cells. For this cytotoxicity test, 1:3 dilutions were used starting with a 3.0 ng/mL TNF sample. A control bacterial lysate, which was employed in the cytotoxicity tests described above, was used as the negative control (non-scTNF expressing bacterial lysate, which was processed identically to the recombinant scTNF proteins).

Figure 4 shows the bioactivity of the employed molecules BEFORE the stability test in mouse fibroblasts MF TNFR1-Fas cells. Soluble human wild-type TNF (TNFhuman) and scTNF dilutions (freshly prepared) were used and diluted out on the cells. Both the soluble control TNF and the scTNF variants after incubation with MF led to their cell death. The control bacterial lysate as expected had no toxic effect. The ED_{50} values (half-maximal effective concentration) were about 0.2 ng/mL for the soluble human wild-type TNF and 0.1 ng/mL for the two employed different scTNF variants. The control bacterial lysate used as the negative control as expected had no toxic effect.

Figure 5 shows the result of a stability test with mouse fibroblasts MF TNFR1-Fas cells. The wild-type TNF and scTNF dilutions, shown in Fig. 5, were hereby incubated for 8 days in a cell incubator before the test in order to be able to evaluate the stability of the samples. A clear decline in the bioactivity of the soluble wild-type TNF (TNFhuman) was measured after 8 days at 37 °C, whereas the activity of both scTNF variants (scTNF3x, scTNF4x; see above for explanation) remained unchanged. An ED_{50} value of about 2 ng/mL (used freshly prepared: 0.2 ng/mL, cf. Fig. 4) was obtained for the wild-type TNF. The scTNF variants with ED_{50} values of about 0.1 ng/mL remained as active as the freshly used samples (cf. Fig. 4). The control bacterial lysate used as the negative control as expected had no toxic effect.

Figure 6 shows the result of a stability test with mouse fibroblasts MF TNFR1-Fas cells. The wild-type TNF and scTNF solutions were now incubated as explained in Fig. 5 for 14 days in a cell incubator. A clear further decline in the bioactivity of the wild-type TNF was measured after 14 days at 37 °C, whereas the activity of the scTNF variants remained approximately the same. An ED_{50} value of about 2.8 ng/mL (freshly prepared 0.2 ng/mL, cf. Fig. 4) was obtained for sTNF; the scTNF variants with 0.13 ng/mL remained virtually as active as the freshly used samples (0.1 ng/mL, cf. Fig. 4). The control bacterial lysate used as the negative control as expected had no toxic effect.

Figure 7 shows the result of a stability test with the human cell line Kym1. Kym1 cells express normal human wild-type TNF receptors and also react cytotoxically to TNF. The experiment was performed similar to the stability tests with mouse fibroblasts according to Figs. 4-6: Soluble wild-type TNF or the scTNF variants (scTNF3x, scTNF4x) were incubated in a serum-containing medium at concentrations of 3.0 to 0.01 ng/mL at 37 °C and 5% CO_2 for different time periods. Next, the functionality of the soluble sTNF or the scTNF variants was tested in cytotoxicity tests on Kym1 cells. Starting with a 3.0 ng/mL TNF samples, 1:3 dilutions were used for the test. The above-described control bacterial lysate was used as the negative control. Wild-type TNF and scTNF dilutions were freshly prepared and used. The result shows that both wild-type TNF and the scTNF variants possessed high cytotoxic activity. The ED_{50} values are about 0.1 ng/mL for the wild-type TNF and about 0.06 ng/mL for the scTNF variants. The control bacterial lysate used as the negative control as expected had no toxic effect.

Figure 8 shows the result of a stability test with the human cell line Kym1. The wild-type TNF and scTNF solutions were hereby incubated for 16 days in a cell incubator. Next, the Kym1 cell cytotoxicity test was performed. A clear decline in the activity of the sTNF was measured after 16 days at 37 °C, whereas the toxicity, i.e., the bioactivity, of the scTNF variants remained approximately the same. An ED_{50} value of about 1.2 ng/mL was obtained for the wild-type TNF. The scTNF variants with 0.06 ng/mL remained as active as the freshly used samples. The control bacterial lysate used as the negative control as expected had no toxic effect.

Figure 9 shows the result of a stability test with the human cell line Kym1. sTNF and scTNF solutions were hereby incubated for 22 days in a cell incubator. A clear further decline in the activity of sTNF was measured after 22 days at 37 °C. The scTNF variants continued to be stable. The control bacterial lysate used as the negative control as expected had no toxic effect.

The following **Table 1** shows the results data from the stability tests according to Figs. 7 and 9 as a comparison:

Table 1: Comparison of the ED_{50} values from Figs. 7 + 9

	ED_{50} freshly titrated (Fig. 7)	ED_{50} after 22 days (Fig. 9)	Loss of activity
sTNF	0.1 ng/mL	1.50 ng/mL	>90%
scTNF	0.06 ng/mL	0.07 ng/mL	Barely detectable

Figure 10 shows the result of a stability test with the human cell line Kym1. Dilutions of 3 ng/mL were titrated out after 16 days at 37 °C. In contrast to the previous Figs. 4-9, in this test a titration curve was prepared starting with a 3 ng/mL TNF dilution stored for 16 days, whereas in the previous shown titration curves the particular dilutions were prepared and then stored for the indicated time period. It can be demonstrated that a comparison of the data from Fig. 8 (similar test with 16 days of incubation) and Fig. 10 shows no major differences. The control bacterial lysate used as the negative control as expected had no toxic effect.

Figure 11 shows the result of a stability test in human serum. To test the stability of wild-type TNF and scTNF in human serum, in a first test batch the soluble wild-type TNF and the scTNF4x variant were diluted in 100% serum and freshly titrated out. An ED_{50} value of 0.004 ng/mL was measured as the result for the wild-type TNF and an ED_{50} value of 0.007 ng/mL for the scTNF variant.

Figure 12 shows the result of a stability test in human serum. To test the stability of wild-type TNF and scTNF in human serum, in another test batch, wild-type TNF and the scTNF4x variant were stored for 8 days at 37 °C in 100% fresh serum that was not heat-inactivated, and then titrated out in Kym1 cells. An ED_{50} value of 0.40 ng/mL was measured as the result for wild-type TNF and an ED_{50} value of 0.03 ng/mL for the scTNF variant. The effects of the 8-day storage in 100% serum were as follows: In comparison with freshly titrated out scTNF, scTNF stored for 8 days exhibited a loss of activity by a factor of about 4.3, whereas for the soluble sTNF there was a dramatic loss of activity by a factor of 100 during storage in 100% serum. The scTNF of the invention, in comparison

with sTNF, accordingly exhibited a much higher bioactivity after an 8-day incubation in serum and thereby proved to be very stable in human serum at physiological temperatures.

In the following **Table 2**, the data of the results from Figs. 11 and 12 are once again presented for clarification.

Table 2: Comparison of the ED_{50} values from Figs. 11 + 12

	ED_{50} freshly titrated (Fig. 11)	ED_{50} after 8 days (Fig. 12)	Loss of activity
Soluble TNF	0.004 ng/mL	0.40 ng/mL	100-fold
Single-chain TNF	0.007 ng/mL	0.03 ng/mL	4.3-fold

Figure 13 shows the result of an analysis by polyacrylamide gel electrophoresis under reducing and denaturing conditions. A silver gel of a purified wild-type TNF and the purified scTNF variants, scTNF3x and scTNF4x, is shown. The samples were each incubated with β-mercaptoethanol (final 5%) at 95 °C for 5 minutes. About 500 ng of sTNF and scTNF4x and about 150 ng of scTNF3x per lane were applied to the silver gel. The result of the silver gel of the scTNF variants separated in 15% SDS-PAGE shows that both scTNF variants under reducing conditions as well had a molecular weight of about 50 kDa, which agrees with their structure. It can also be stated that the different amounts of the applied proteins scTNF3x and scTNF4x had no effect on the result. This confirms the stability of the proteins or polypeptides of the invention under reducing and denaturing conditions. The result for sTNF, in contrast, shows that the protein breaks down into its monomers of about 17 kDa.

Figure 14 shows the result of a stability test under reducing and denaturing conditions. In this test, a Western Blot of samples, separated in 15% SDS-PAGE, of wild-type TNF and the scTNF variants scTNF3x and scTNF4x was performed. Two parallel batches were carried out in which the samples in one batch were each incubated with β-mercaptoethanol (final 5%) at 95 °C for 5 minutes, whereas no β-mercaptoethanol

incubation occurred in the other batch. The detection after the electrophoresis run was performed with an anti-TNF antibody. It is evident as a result that the antibody specifically detected the protein of both scTNF variants in clear bands at about 50 kDa. For the wild-type TNF, the protein was specifically detected at about 17 kDa. This again confirms the stability of the scTNF3x and scTNF4x variants of the invention under reducing and denaturing conditions and also coincides as expected with the results from Fig. 13.

Figure 15 shows the result of an IkappaB degradation assay. IkappaB (I-κB) is an inhibitor of the transcription factor nuclear factor kappa B (NF-κB), and as is generally known is caused to degrade after addition of TNF, as a result of which NF-κB is activated. To determine the degradation of I-κB, cell lysates were prepared 0, 30, and 60 minutes after stimulation with 10 ng/mL each of wild-type TNF, scTNF3x, and scTNF4x and then analyzed using the Western Blot assay with I-κB-specific antibodies. The result shows that the transient degradation of I-κB of both wild-type TNF and the two scTNF variants was induced, whereby the reaction behavior of the scTNF variants corresponds to that of the wild-type TNF. The control bacterial lysate used as the negative control (as described above, a non-scTNF expressing bacterial lysate, which was processed identically to the recombinant scTNF-proteins) as expected had no effect on the I-κB degradation.

Figure 16 shows the result of a JNK assay. JNK (c-jun N-terminal kinase) is a stress-induced kinase, which as is known is very highly activated by TNF. After stimulation of Kym-1 cells for 0, 30, or 60 minutes with 10 ng/mL each of wild-type TNF, scTNF3x, and scTNF4x, cell lysates were prepared and the JNK activity was determined by immunoprecipitation of JNK with JNK-specific antibodies and subsequent kinase assay with GST c-Jun as the substrate. The control bacterial lysate used as the negative control (as described above) as expected did not activate JNK kinase. The result shows that both scTNF variants and wild-type TNF activated JNK, whereby the reaction behavior of the scTNF variants corresponded to that of the wild-type TNF.

Figure 17 shows the result of an Electrophoretic Mobility Shift Assay (EMSA). Another typical test to determine the activity of TNF is the translocation of the transcription factor NF-κB into the cell nucleus after induction of the I-κB degradation. For this purpose, cell nucleus preparations of non-stimulated KYM-1 control cells (zero minutes of stimulation) or of stimulated cells (30 and 60 minutes of stimulation) were made. The stimulation of the cells occurred in each case with wild-type TNF, scTNF3x, and scTNF4x. The transcription factor NF-κB, translocated into the cell nucleus, was detected with the use of NF-κB-specific, radioactively labeled oligonucleotides. The result shows that NF-κB was translocated into the cell nucleus. The reaction behavior of the scTNF variants corresponds to that of the wild-type TNF.

Figure 18 shows an exemplary construct scheme of the polypeptides of the invention, presented for TNF (as a member of the TNF ligand family). The designations have the following meanings:

Constructs AI/AII and BI/II have an optimized codon usage for the expression of proteins in *E. coli*, and represent molecules which are each linked with GlyGlyGlySer-3x linker or 4x linker. All molecules have a histidine tag N-terminally for easier purification; the molecules BI/BII in addition have a short amino acid chain with a cysteine residue for directional covalent linkage.

Constructs C to H are suitable for expression of the proteins in eukaryotic cells; they have the appropriate leader peptide sequences N-terminally.

sc = is the abbreviation for single chain,

cys = designates an N-terminal peptide linker with internal cysteine for covalent coupling,

$L1_{long}$ or $L2_{long}$ = designates linker 1 or 2, each with the amino acid sequence (GGGS-GGGS-GGGS-GGGS) or $(GGGS)_4$,

$L1_{short}$ or $L2_{short}$ = designates linker 1 or 2, each with the amino acid sequence (GGGS-GGGS-GGGS) or $(GGGS)_3$,

Leader peptide sequence = is the amino acid sequence for the secretion of the protein from eukaryotic (host) cells.

scFv10 = stands for the sequence of the single-chain (scFv) antibody fragment 40, specific for the tumor stroma antigen FAP,

AMAIZe = is the abbreviation for "Antibody-Mediated Apoptosis-Inducing Cytokines" and

His/Flag tag = stands for the peptide sequence for an affinity purification of the expressed proteins.

Figure 19 shows the nucleic acid sequence and the corresponding amino acid sequence of scTNF-L_{short} (construct A-II)

Features of construct A-II

- His tag peptide sequence for affinity purification of the formed protein: amino acids (abbreviated as "AA" below in Figs. 19 through 26) AA 5-10, nucleotide (abbreviated as "NT" in Figs. 19 through 26 below) NT 13-30
- Sequence of the human TNF module1 (extracellular domain, AA 79-181, of the natural human TNF molecule, sequence with optimized E. coli codon usage): AA 11-169, NT 31-507
- Sequence of the $(GGGS)_3$-linker1: AA 170-181, NT 508-543
- Sequence of the human TNF module2 (extracellular domain, AA 79-181, of the natural human TNF molecule, sequence with optimized E. coli codon usage): AA 182-335, NT 544-1005
- Sequence of the $(GGGS)_3$-linker2: AA 336-347, NT 1006-1041

- Sequence of the human TNF module3 (extracellular domain, AA 79-181, of the natural human TNF molecule, sequence with optimized E. coli codon usage): AA 348-501, NT 1042-1503
- Stop codon: NT 1504-1506

Figure 20 shows the nucleic acid sequence and the corresponding amino acid sequence of cys-scTNF-L_{short} (construct B-II)

Features of construct B-II

- Amino acid cysteine for covalent coupling: AA 9, NT 25-27
- His tag peptide sequence for affinity purification of the formed protein: AA 15-20, NT 43-60
- Sequence of the human TNF module1 (extracellular domain of the natural human TNF molecule, sequence with optimized E. coli codon usage): AA 21-181, NT 61-543
- Sequence of the $(GGGS)_3$-linker1: AA 182-193, NT 544-579
- Sequence of the human TNF module2 (extracellular domain of the natural human TNF molecule, sequence with optimized E. coli codon usage): AA 194-347, NT 580-1041
- Sequence of the $(GGGS)_3$-linker2: AA 348-359, NT 1042-1077
- Sequence of the human TNF module3 (extracellular domain of the natural human TNF molecule, sequence with optimized E. coli codon usage): AA 360-513, NT 1078-1539
- Stop codon: NT 1540-1542

Figure 21 shows the nucleic acid sequence and the corresponding amino acid sequence of scFasL (construct C)

Features of construct C

- Leader peptide sequence for secretion of the protein in eukaryotic cells: AA 1-15, NT 1-45
- Flag tag peptide sequence for affinity purification of the formed protein: AA 19-26, NT 55-78
- Sequence of the human FasL module1 (extracellular domain, AA 139-281, of the natural human FasL molecule): AA 30- 173, NT, 90-519
- Sequence of the $(GGGS)_4$-linker1: AA 174-189, NT 520-567
- Sequence of the human FasL module2 (extracellular domain, AA 139-281, of the natural human FasL molecule): AA 190- 332, NT 568-996
- Sequence of the $(GGGS)_4$-linker2: AA 333-348, NT 997-1044
- Sequence of the human FasL module3 (extracellular domain, AA 139-281, of the natural human FasL molecule): AA 349-491, NT 1045-1473
- Stop codon: NT 1474-1476

Figure 22 shows the nucleic acid sequence and the corresponding amino acid sequence of scTRAIL (construct D)

Features of construct D

- Leader peptide sequence for secretion of the protein in eukaryotic cells: AA 1-15, NT 1-45
- Flag tag peptide sequence for affinity purification of the formed protein: AA 19-26, NT 55-78
- Sequence of the human TRAIL module1 (extracellular domain, AA 95-281, of the natural human TRAIL molecule): AA 30-216, NT 88-648
- Sequence of the $(GGGS)_4$-linker1: AA 217-232, NT 649-696
- Sequence of the human TRAIL module2 (extracellular domain, AA 95-281, of the natural human TRAIL molecule): AA 233-419, NT 697-1257

- Sequence of the $(GGGS)_4$-linker2: AA 420-435, NT 1258-1305
- Sequence of the human TRAIL module3 (extracellular domain, AA 95-281, of the natural human TRAIL molecule): AA 436-622, NT 1306-1866
- Stop codon: NT 1861-1863

Figure 23 shows the nucleic acid sequence and the corresponding amino acid sequence of scTNF (construct E)

Features of construct E

- Leader peptide sequence for secretion of the protein in eukaryotic cells: AA 1-15, NT 1-45
- Flag tag peptide sequence for affinity purification of the formed protein: AA 19-26, NT 55-78
- Sequence of the human TNF module1 (extracellular domain of the natural human TNF molecule): AA 30-184 , NT 88-552
- Sequence of the $(GGGS)_4$-linker1: AA 85-200, NT 553-600
- Sequence of the human TNF module2 (extracellular domain of the natural human TNF molecule): AA 201-355, NT 601-1065
- Sequence of the $(GGGS)_4$-linker2: AA 356-371, NT 1066-1113
- Sequence of the human TNF module3 (extracellular domain of the natural human TNF molecule): AA 372-526, NT 1114-1581
- Stop codon: NT 1799-1581

Figure 24 shows the nucleic acid sequence and the corresponding amino acid sequence of scFasL-AMAIZe (construct F)

Features of construct F

- Leader peptide sequence for secretion of the protein in eukaryotic cells: AA 1-19, NT 1-57

- Sequence of the single-chain (scFv) antibody fragment 40 specific for the tumor stroma antigen FAP: AA 20-267, NT 58-801
- Flag tag peptide sequence for affinity purification of the formed protein: AA 278-285, NT 832-855
- Sequence of the human FasL module1 (extracellular domain, AA 139-281, of the natural human FasL molecule): AA 290-432, NT 868-1296
- Sequence of the $(GGGS)_4$-linker1: AA 433-448, NT 1297-1344
- Sequence of the human FasL module2 (extracellular domain, AA 139-281, of the natural human FasL molecule): AA 449-591, NT 1345-1773
- Sequence of the $(GGGS)_4$-linker2: AA 592-607 , NT 1774-1821
- Sequence of the human FasL module3 (extracellular domain, AA 139-281, of the natural human FasL molecule): AA 608-750, NT 1822-2250
- Stop codon: NT 2251-2253

Figure 25 shows the nucleic acid sequence and the corresponding amino acid sequence of scTRAIL-AMAIZe (construct G)

Features of construct G

- Leader peptide sequence for secretion of the protein in eukaryotic cells: AA 1-19, NT 1-57
- Sequence of the single-chain (scFv) antibody fragment 40 specific for the tumor stroma antigen FAP: AA 20-267, NT 58-801
- Flag tag peptide sequence for affinity purification of the formed protein: AA 278-285, NT 832-855
- Sequence of the human TRAIL module1 (extracellular domain, AA 95-281, of the natural human TRAIL molecule): AA 289-475, NT 865-1426
- Sequence of the $(GGGS)_4$-linker1: AA 476-491, NT 1427-1476
- Sequence of the human TRAIL module2 (extracellular domain, AA 95-281, of the natural human TRAIL molecule): AA 492-678, NT 1477-2034

- Sequence of the $(GGGS)_4$-linker2: AA 679-694, NT 2035-208 [sic]
- Sequence of the human TRAIL module3 (extracellular domain, AA 95-281, of the natural human TRAIL molecule): AA 695-, NT 2083-2643
- Stop codon: NT 2644-2646

Figure 26 shows the nucleic acid sequence and the corresponding amino acid sequence of scTNF-AMAIZe (construct H)

Features of construct H

- Leader peptide sequence for secretion of the protein in eukaryotic cells: AA 1-19, NT 1-57
- Sequence of the single-chain (scFv) antibody fragment 40 specific for the tumor stroma antigen FAP: AA 20-267, NT 58-801
- Flag tag peptide sequence for affinity purification of the formed protein: AA 278-285, NT 832-855
- Sequence of the human TNF module1 (extracellular domain, AA 79-181, of the natural human TNF molecule): AA 289-443, NT 865-1329
- Sequence of the $(GGGS)_4$-linker1: AA 444- , NT 1330-1377
- Sequence of the human TNF module2 (extracellular domain, AA 79-181, of the natural human TNF molecule): AA 460-614, NT 1378-1842
- Sequence of the $(GGGS)_4$-linker2: AA 615-630 , NT 1843-1890
- Sequence of the human TNF module3 (extracellular domain, AA 79-181, of the natural human TNF molecule): AA 631-785, NT 1891-2353
- Stop codon: NT 2356-2358

Figure 27 shows the pharmacokinetics of human wild-type TNF and human scTNF (cf. Example 2). The data in Fig. 27 show a clear increase in the *in vivo* half-life of the scTNF variants. A clearly increased duration of action *in vivo* is expected for scTNF compared

with TNF, which thereby emphasizes the value of scTNF, particularly of scTNF-L_2, as a potential therapeutic agent.

Figure 28 shows CysHis-scTNF coupled covalently to particles (silica). This covalently coupled CysHis-scTNF is bioactive and possesses the special activity of membrane-bound TNF; i.e., it activates TNFR2. Fig. 28 shows that cells from mouse fibroblasts, which are transfected with the construct TNFR2-Fas and were treated with serial dilutions of the indicated reagents, are totally resistant to soluble wtTNF. After covalent coupling of reduced CysHis-scTNF to silica microparticles (beads) according to established protocols (DPA 2001, No. DE 10144252), these cause a strong cytotoxic response (circle), like a positive control consisting of CysHis-scTNF and a TNFR2 cross-linking antibody, mAk 80M2 (triangle). Cys-His scTNF, which is not coupled, as expected shows no activity on TNFR2-positive cells (squares).

The present invention will be illustrated below by the examples:

Examples

Example 1: Preparation of different polypeptide constructs of the invention

All clonings were performed according to standard protocols. The conditions for these are given below.

Standard PCR:

60 ng of template, 0.5 µL of 100 µM primer, 1 µL of 10 mM dNTPs, 5 µL of 10x buffer, and 2 U of Taq polymerase were amplified in a reaction volume of 50 µL using the following PCR program. Denaturation: 94 °C 3 minutes; 15 cycles: denaturation 94 °C 30 seconds, annealing 55 °C 30 seconds, elongation 72 °C 90 seconds; final elongation: 72 °C 7 minutes.

Digestion of PCR products:

The PCR product was purified over an agarose gel and eluted and then digested with the appropriate restriction enzymes (see specific instructions) in a 40-µL reaction batch at an optimal cleavage temperature (indicated by the manufacturer) for 2 hours.

Digestion of vectors:

1 µg of vector was digested with 5 U of the appropriate restriction enzymes in a 20-µL reaction volume for 2 hours at an optimal cleavage temperature (this depends on employed enzyme and is indicated by the manufacturer). To dephosphorylate the vectors, 10 U of alkaline phosphatase was added for 1 hour to the reaction digest.

Fill-in reaction:

The batch from a vector digestion was combined with 33 µM dNTPs and 1 U/1 µg of DNA Klenow fragment of DNA polymerase I and incubated for 15 minutes at 25 °C. This reaction was stopped with 10 mM EDTA for 20 minutes at 75 °C.

Ligation:

Vector and insert were ligated at a molar ratio of 1:5 together with 400 U of ligase in a 10-µL volume overnight at 16 °C.

I. Preparation of $scTNF_{human}$ (scTNF) with 4x or 3x linkers:

Two scTNF variants were prepared, which differ in the length of the peptide linker between the individual modules. Primers with a 4x or 3x peptide-linker sequence were used: $linker_{long}$ with the $(GGGS)_4$ sequence or $linker_{short}$ with the $(GGGS)_3$ sequence. The produced constructs accordingly contained either only two 4x linkers (L1 or L2 with $(GGGS)_4$ - designated as "long") or only two 3x linkers (L1 or L2 with $(GGGS)_3$ - designated as "short").

1. A standard PCR was run with primers V and I or II (for $linker_{long}$) and the pQE-9 vector with a TNF module (pQE9-HisTNF) as the template. The vector bears a His tag sequence for later affinity purification of the produced protein.

2. The obtained PCR product I was then digested with 20 U of the appropriate restriction enzymes StuI and HindIII at 37 °C. The same digestion and a dephosphorylation reaction were performed with the pQE9-HisTNF vector, and the PCR product I was inserted into the pQE9-HisTNF vector by ligation. The result of this step was a His tag - TNF module1 with a $linker1_{short\ or\ long}$, or the following construct in the pQE9 vector:

EcoRI - His tag - TNF module1 - $linker1_{short\ or\ long}$ - BamHI

3. PCR product II was produced by another PCR with primers III and I or II (for $linker_{long}$) and the pQE9-HisTNF vector as the template. PCR product II was then cut each time with 20 U of the restriction enzymes EcoRI and HindIII and inserted into the pQE9-HisTNF vector, which was cut with the same enzymes and dephosphorylated. The result of this cloning was a pQE9 vector, which was as follows:

TNF module2 - linker2$_{short\ or\ long}$ - BamHI

This construct has no sequence for a His tag before the TNF sequence.

4. The cloned PCR product II from step 3 was first cut from the vector with the restriction enzyme Hind III and then partially with BamHI (1 U/µg of DNA). The pQE9-His tag TNF module1 - linker1$_{short\ or\ long}$ vector was also cut sequentially with the restriction enzymes BamHI and HindIII, dephosphorylated, and the PCR product II ligated into this vector. The result was a pQE9 vector with following construct:

His tag - TNF module1 - linker1$_{short\ or\ long}$ TNF module2 - linker2$_{short\ or\ long}$.

5. Another PCR was performed under standard conditions with the pQE9-HisTNF vector as the template and primers III and IV, and the obtained PCR product III was sequentially digested with restriction enzymes BamHI (40 U) and HindIII (40 U). This fragment was then ligated into a pBluescript SKII vector, which was also cut with the restriction enzymes BamHI and HindIII. The result of this cloning was a pBluescript SKII vector, which contains the TNF module 3 without linkers.

6. The pQE9 vector with the **His tag - TNF module1 - linker1$_{short\ or\ long}$ TNF module2 - linker2$_{short\ or\ long}$** construct was cut with the restriction enzyme EcoRI; this vector was then treated with the Klenow fragment of DNA polymerase I from E. coli, to carry out a fill in. After this step, a partial restriction digestion was carried out with the enzyme BamHI (1 U/µg DNA).

7. Parallel to Step 6, the pBluescript SKII vector, which contains the TNF module 3, was cut with the restriction enzyme XbaI; this vector was then treated with the Klenow fragment of DNA polymerase I from E. coli, to carry out a fill in. After this step, a second restriction digestion was carried out with the enzyme BamHI under standard conditions with additional dephosphorylation.

8. The fragment obtained by the restriction digestion from Step 6 was then ligated into the linear vector generated in Step 7. The constructs were in reverse order as follows: HindIII - TNF module3 - linker2$_{\text{short or long}}$ - TNF module2 - linker2$_{\text{short or long}}$ - TNF module1 - His tag - EcoRI

9. The reverse TNF construct from Step 8 was cut from the pBluescript SKII vector with the restriction enzymes EcoRI and HindIII and ligated into the pQE9-HisTNF vector, which was treated with the same enzymes and dephosphorylated. As a result, the following construct with the complete scTNF in correct orientation formed:
EcoRI - His Tag - TNF module1 - linker1$_{\text{short or long}}$ - TNF module2 - linker2$_{\text{short or long}}$ - TNF module3 - HindIII

10. To prepare an scTNF with an N-terminal cysteine, the oligos cys-scTNF VI and VII were annealed (20 µL each of 100 µM oligo VI or VII were heated together for 5 minutes at 95 °C and slowly allowed to cool to room temperature), and oligo1 was formed in this way. The construct from Step 9 was digested with the restriction enzymes EcoRI and BbsI and the oligo1, which has the same cut sites, was ligated into the vector. Alternatively, the cysteine was inserted via PCR mutagenesis. The result of this cloning was the following construct:
EcoRI - Cysteine-His Tag - TNF module1 - linker1$_{\text{short or long}}$ - TNF module2 - linker2$_{\text{short or long}}$ - TNF module3 - HindIII

All constructs were verified by sequencing.

The expression was carried out in the *E. coli* strain XL-1 blue. The purification of the expressed scTNF variants occurred with use of chromatographic methods (His tag affinity and anion exchange chromatography).

The sequences of the employed primers are given below:

Peptide linker sequences at the protein level

3x GGGS-linker (short) = $(GGGS)_3$: GGGS GGGS GGGS

4x GGGS-linker (long) = $(GGGS)_4$: GGGS GGGS GGGS GGGS

Peptide linker sequences at the nucleotide level

3x GGGS-linker (short): 5'GGT GGC GGT TCT GGT GGC GGT TCT GGT GGC GGA TCC3'

4x GGGS-linker (long): 5'GGT GGC GGT TCT GGT GGC GGT TCT GGT GGC GGT TCT GGT GGC GGA TCC3'

Primers for the clonings

scTNF Primer I

5'- TCG ATT AAG CTT CCC GGG GGA TCC GCC ACC AGA ACC GCC ACC AGA ACC GCC ACC CAG AGC GAT GAT ACC GAA GTA AAC CTG ACC -3'

scTNF Primer II

5'- ATC GAT TAA GCT TCC CGG GGG ATC CGC CAC CAG AAC CGC CAC CAG AAC CGC CAC CAG AAC CGC CAC CCA GAG CGA TGA TAC CGA AGT AAA CCT GAC C -3'

scTNF Primer III

5'- CCC CGA ATT CGG ATC CTC TTC TCG TAC CCC GTC TGA CAA ACC G -3'

scTNF Primer IV

5'- GGG GGG GAA GCT TAT CGA TAG TTA GAT ATC ATC ACA GAG CGA TGA TAC CGA AG -3'

scTNF Primer V

5'- CCT GTA CCT GAT CTA CTC CCA GGT TCT GTT CAA AGG CCA GG -3'

Oligo for CysHis insertion:

cys-scTNF Primer VI

AAT TCA TTA AAG AGG AGA AAT TAA CTA TGG GAG AGC TCA TCG AAG GTC GCT GCG CCG GTG GAT CTG GTC ATC ATC ATC ACC ATC ACG GCT CAG ACG G

cys-scTNF Primer VII

CGC TCC GTC TGA GCC GTG ATG GTG ATG ATG ATG ACC AGA TCC ACC GGC GCA GCG ACC TTC GAT GAG CTC TCC CAT AGT TAA TTT CTC CTC TTT AAT G

II. Preparation of scFasL in pcDNA3 and AMAIZe constructs:

The standard conditions given in Example 1 were used for the following clonings.

A. Generation of the HA signal in pcDNA3

1. Digestion of the pcDNA3 vector with KpnI and NotI.
2. Preparation of the HA oligo with the KpnI-HA-signal-NotI sequence by annealing of the primers HA-IF and HA-IIR for the leader peptide sequence:
3. Ligation of the HA oligo (contains KpnI and NotI cut sites) into the pcDNA3 vector

B. Preparation of scFasL in the pcDNA3(+) vector

1. PCR with primers FasL#1F and FasL#2R on the template FasL-AMAIZE vector. The preparation of this type of constructs is described in German Patent Application DE 10045591.3, which is herewith incorporated in its entirety in the disclosure of the present invention. The product of this PCR 1 was a **NotI - Flag tag - FasL module1 - linker1 – BamHI - XbaI** construct.

2. Using the NotI and XbaI restriction cut sites, this construct was cloned in the pcDNA3-HA sequence vector with the same enzymes, so that the following construct forms:

HA sequence - Flag tag - FasL module1 - linker1 - BamHI - XbaI

3. The following PCR product 2 was generated with the use of another PCR on the template FasL-AMAIZE vector and the primers FasL#3 and FasL#1:
blunt end - FasL module2 - linker2 - BamHI - XbaI

4. In the next step, the pcDNA3 vector from Step 2 was digested with BamHI; this cut site was filled with the Klenow enzyme and then cut with XbaI, so that a "blunt end" and a "sticky end" formed. PCR product 2 was then cloned in this thus modified vector, with the formation of the following construct:
HA sequence - Flag tag - FasL module1 - linker1 - Fasl module2 - linker2 - BamHI - XbaI

5. For the third module, another PCR was performed with the template FasL-AMAIZE vector and the primers FasL#4 and FasL#5. The formed PCR product was then digested with the restriction enzymes BamHI and XbaI and ligated into the vector, cut with the same enzymes, from Step 4. The result of this cloning was the following construct in the pcDNA3 vector:
HA sequence - NotI - Flag tag - FasL module1 - linker1 - FasL module2 - linker2 - FasL module3 - stop - XbaI.

To prepare the scFasL- or scTNF-AMAIZe constructs, the corresponding scFasL or scTNF was digested with the restriction enzymes NotI or EcoRI and XbaI and the inserts were inserted as cassettes into the corresponding AMAIZe vectors (see German Patent Application DE 10045591.3), whereby these vectors were also cut with the enzymes NotI or EcoRI and XbaI. The following constructs were prepared in this way:
Leading peptide - scFv40 - Flag tag - FasL module1 - linker1 - FasL module2 - linker2 - FasL module3
Leading peptide - scFv40 - Flag tag - TNF module1 - linker1 - TNF module2 - linker2 - TNF module3

The sequences of the employed primers are given below:

FasL#1R:
5'ATCGATTTCTAGACCCGGGGGATCCGCCACCAGAACCGCCACCAGAACCGCC
ACCAGAACCGCCACCGAGCTTATATAAGCCGAAAAACGTCTGAGATTC3'

FasL#2F:
5'GGGGTAGCGGCCGCGCTGTCGACGATTACAAAGAC3'

FasL#3F:
5'AGAAAAAAAGGAGCTGAGGAAAGTGG3`

FasL#4F:
5'GGGGCGGATCCGAAAAAAAGGAGCTGAGGAAAGTGG3'

FasL#5R:
5'GGGGCCTCTAGAATCGATGGTCAGAGCTTATATAAGCCGAAAAACGTCTG3'

HA-IF
5'CGCCAT GGCTATCATC TACCTCATCC TCCTGTTCAC CGCTGTGCGG
GGAGC3'

HA-IIR
5'GGC CGC TGC CCC GCA CAG CGG TGA ACA GGA GGA TGA GGT AGA TGA
TAG CCA TGG CGG TAC3'

III. scTRAIL cloning and preparation of scTRAIL AMAIZe constructs

The standard conditions given in Example 1 were used for the following clonings.

1. PCR with primers TRAIL#1 and TRAIL#2 on the template pcDNA3-sc40-TRAIL (see German Patent Application DE 10045591.3). PCR product 1 was cut with EcoRI

and XbaI and ligated into the pcDNA3-scFasL vector digested with the same restriction enzymes. This digestion deleted the FasL sequence, whereby the HA and Flag tag sequence was retained and the following construct was now formed:

HA sequence - Flag tag - TRAIL module1 - linker1 - BamHI - XbaI

2. Using the primers TRAIL#1R and TRAIL#2F, PCR product 2 was generated with the template TRAIL-AMAIZe (see German Patent Application DE 10045591.3). This was cut only with XbaI, a blunt end and a sticky end forming. The construct from Step 1 was digested with BamHI and then treated with the Klenow enzyme, so that the ends were filled. After this, an XbaI digestion was performed and the PCR product 2 was cloned in this vector. The result was the following construct:

   HA sequence - Flag tag -TRAIL module1 - linker1 - TRAIL module2 - linker2 - BamHI - XbaI

3. For the cloning of TRAIL module 3, a PCR was carried out with the primers TRAIL#4 and TRAIL#5 on the template TRAIL-AMAIZe, the product was then digested with BamHI and XbaI, and cloned in the construct from Step 2 - also digested with BamHI and XbaI, as a result of which the following construct formed:

   HA sequence - Flag tag -TRAIL module1 - linker1 -TRAIL module2 - linker2 - TRAIL module3 Stop - XbaI

For the preparation of the scTRAIL-AMAIZe constructs, the specific scTRAIL vectors were digested with the restriction enzymes NotI or EcoRI and XbaI and the inserts were inserted as cassettes into the corresponding AMAIZe vectors (see German Patent Application DE 10045591.3), whereby these vectors were also cut with the enzymes NotI or EcoRI and XbaI. The following constructs were prepared in this way:

HA svFv40 - Flag tag - TRAIL module1 - linker1 -TRAIL module2 - linker2 - TRAIL module3

The sequences of the employed primers are given below:

Primers for the scTRAIL cloning

TRAIL#1R:
5'ATCGATTTCTAGACCCGGGGGATCCGCCACCAGAACCGCCACCAGAACCGCC
ACCAGAACCGCCACCGCCAACTAAAAAGGCCCCGAAAAAACTGGCTT-
CATGGTC3'

TRAIL#2F:
5'GGGGTAGAATTCGGAACCTCTGAGGAAACCATTTCTACAGTTCAAG3'

TRAIL#3F:
5'AACCTCTGAGGAAACCATTTCTACAG3'

TRAIL#4F:
5'GGGGCGGATCCACCTCTGAGGAAACCATTTCTACAG3'

TRAIL#5R:
5'GGGGCCTCTAGAATCGATGGTCAGCCAACTAAAAAGGCCCCGAAAAAACTGG
C3'

Example 2: Pharmacokinetics of human wild-type TNF and human scTNF

Six-week-old Balb/c mice were injected i.v. with 12 µg of TNF or scTNF (3 mice in each case). Blood was taken every 45 minutes and collected, and the concentration of TNF in the serum was determined using a human TNF-specific ELISA kit. The data in Fig. 27 show a clear increase in the *in vivo* half-life of the scTNF variants. It is therefore expected for scTNF that it has a clearly increased duration of biological action in vivo, which thereby emphasizes the value of scTNF as a potential therapeutic agent.

Example 3: CysHis-scTNF coupled covalently to particles (silica)

Mouse fibroblasts, transfected with the construct TNFR2-Fas, were treated with serial dilutions of the indicated reagents. These cells are completely resistant to soluble wtTNF.

After covalent coupling of reduced CysHis-scTNF to silica microparticles (beads) according to established protocols (DPA 2001, No. DE 10144252), these cause a strong cytotoxic response (circle), like a positive control consisting of CysHis-scTNF and a TNFR2 cross-linking antibody, mAk 80M2 (triangle). Cys-His scTNF, which is not coupled, as expected shows no activity on TNFR2 positive cells (squares). Covalently coupled CysHis-scTNF is bioactive and possesses the special activity of membrane-bound TNF; i.e., it activates TNFR2.

Example 4: Comparison of standard recombinant human (rh)TNF and scTNF in *in vivo* tumor necrosis models and *in vitro* L929 cytotoxicity activity

Mice: C3H/HeJ (female), 17-19 g, from Charles River

Tumor cells: CFS-1 methylcholanthrene-induced fibrosarcoma cell line derived from C3H/HeN mouse (reference: Hafner M., P. Orosz, A. Krüger, and D.N. Männel. 1996 TNF promotes metastasis by impairing natural killer cell activity. *Internat. J. Cancer* 66:388-392).

Tumor necrosis experiment: The mice received 1.6 x 10^7 CFS-1 cells in 50 µL of medium (RPMI, 10% FCS) intradermally in the back; the tumors were allowed to grow for 12 days until they reached a size of about 5-6 mm in diameter, before the intraperitoneal injection of TNF (10 µg per mouse) in 200 µL of PBS or PBS alone as control. The tumor size was measured daily and examined grossly. The mice were sacrificed on Day 6 after the treatment and the tumors were removed for histology. The tumors were excised, fixed overnight in 4% PBS-buffered formalin, and embedded in paraffin. Equatorial vertical sections (4 µm) were stained with hematoxylin and eosin, and examined microscopically for necrosis (as described, e.g., in: Lucas R. et al., 2001, Int J Cancer, 91:543-549).

TNF: rhTNF specific activity 6.6 x 10^6 U/mg (48-hour L929 test without Act D)
scTNF

In vitro experiment, LD_{50} activity in the L929 cytotoxicity assay with Act D for:

rhTNF = 391 pg/mL

scTNF = 39 pg/mL

(tested with the same TNF samples, which were used for *in vivo* experiments)

scTNF in this in vitro experiment shows a 10-fold increased activity.

In vivo tumor necrosis experiment:

Group	n	Tumor diameter		Necrosis		
		d0	d4	gross*	microscopic**	
					<5%	>10%
PBS	6	5.2 + 1	7.4 + 0.5	1	2	0
rhTNF	7	5.2 + 0.9	6.6 + 1.7	3	6	1
scTNF	7	5.9 + 0.5	7.3 + 0.3	5	0	7

* = grossly clearly discernible superficial necrosis

** = on microscopic examination, central hemorrhagic necrosis, <5% or >10% of the tumor tissue

Conclusion:

After 4 days of treatment with single doses, there was no difference in the tumor size (for an overview on TNF as a tumor therapeutic agent, see, e.g., Eggermont et al, Lancet Oncol. 4, 429 (2003)).

rhTNF induced small hemorrhagic necrosis (<5% of the tumor area), visible grossly in only 3/7 of the animals.

scTNF induced larger hemorrhagic necroses (> 10% of the tumor area) in all tumors (7/7), 5/7 of which can be seen grossly.

scTNF >> rhTNF in relation to tumor cytotoxicity *in vitro* and induction of necrosis.